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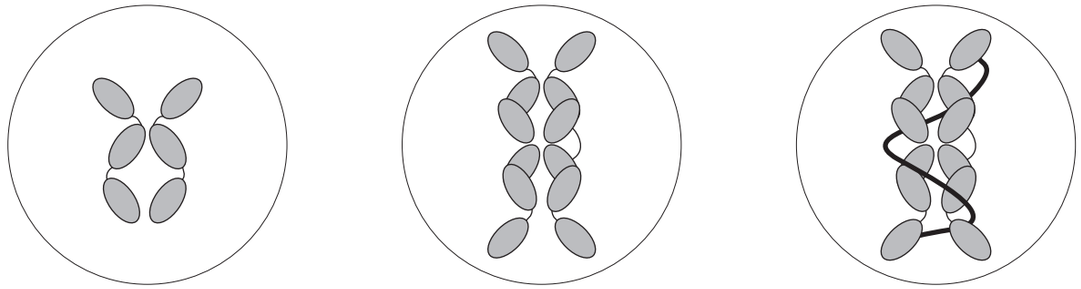

Oral passive immunisation of weaned piglets against F4-positive enterotoxigenic *Escherichia coli* by in seed produced antibodies

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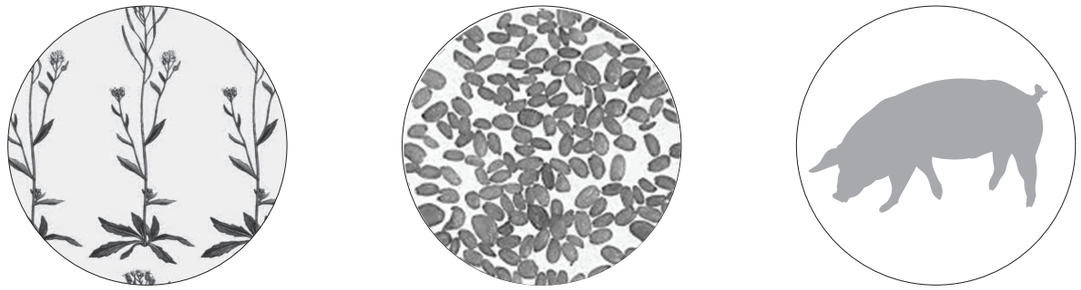

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Oral passive immunisation of weaned piglets against F4-positive enterotoxigenic *Escherichia coli* by in seed produced antibodies

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Thesis submitted in partial fulfillment of the requirements
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Table of Contents

Frequently used abbreviations

2PM-PCR	Two-Primer Multiplex-Polymerase Chain Reaction*
2S2	signal peptide sequence of the 2S2 seed storage protein
3'arc6H5I	600 bp long 3' arceline terminator bearing regulatory sequence
3'arc5I	3' arceline terminator bearing regulatory sequence
3'nos	nopaline synthase terminator
3'ocs	octopine synthase terminator
Ab	Antibody
ADCC	Antibody-Dependant Cell-mediated Cytotoxicity
att sites	attachment sites within Gateway vectors (Invitrogen)
bar	phosphinothricin herbicide resistant gene
BiP	Binding protein (an ER resident chaperon)
BLAST	Basic Local Alignment Search Tool
bp	base pair
BTV	Blue Tongue Virus
CaMV	Cauliflower mosaic virus
CDR	Compliment Determining Region
cGMP	current Good Manufacturing Practices
CTAB	Cetyltrimethylammoniumbromid
DARPA	Defence Advance Research Project Agency (of USA)
dIgA	dimeric Immunoglobulin A
ELISA	Enzyme-linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
ETEC	Enterotoxigenic *Escherichia coli*
$F18^+$ETEC	F18 fimbriae bearing enterotoxigenic *Escherichia coli*
$F4^+$ETEC	F4 fimbriae bearing enterotoxigenic *Escherichia coli*
F4R	F4 receptor (receptor on villous surface to which $F4^+$ETEC attaches)
Fab	Fragment antigen binding
FaeG	major adhesin of the F4 fimbriae

Fc	Fragment crystallisable
FCR	Feed Conversion Ratio
FcRn	Fc (Fragment crystallisable) neonatal receptor
FcγR	Fc receptor for gamma globulins
FDA	Food and Drug administration
GM	Genetically modified
GMO	Genetically modified organism
HCAb	Heavy chain only antibody of camelids
HIV	Human Immunodeficiency Virus
HRSV/RSV	Human Respiratory Syncytial Virus / Respiratory Syncytial Virus
HSV	Herpes simplex virus
ICAM1	Intercellular Adhesion Molecule 1
Ig	Immunoglobulin
Ig-NAR	Immunoglobulin-Novel Antigen Receptor
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
J chain	Joining Chain
KDEL	(Lys-Asp-Glu-Leu) a C-terminal ER retention peptide
LB	Left border
mAb	Monoclonal antibodies
MBP	Maltose binding protein
mIgA	monomeric Immunoglobulin A
NHL	Non-Hodgkin's lymphomas
nptII	neomycin phosphotransferase II gene
OD	Optical Density
Omega leader	tobacco mosaic viral UTR
PBS	Phosphate Buffer Saline
PIgR	Polymeric Immunoglobulin Receptor
Pnos	nopaline synthase gene promoter

PoLa	Porcinised Lama antibodies*
Pphas	Phaseolin promoter
PPT	phosphinothricin
PWD	Post-weaning diarrhoea
RB	Right border
RFLP	Restriction Fragment Length Polymorphism
SC	Secretory Component
ScFv	Single chain variable fragment
ScFv-Fc	Single chain variable fragment-(fused to)-fragment crystallisable
SEKDEL	Ser-Glu-Lys-Asp-Glu-Leu (a C-terminal ER retention peptide)
SIgA	Secretory Immunoglobulin A
sSIgA	simplified Secretory Immunoglobulin A*
STD	Sexually Transmitted Disease
TSP	Total Soluble Protein
UTR	Untranslated Regions
V-NAR	Variable domain of a novel antigen receptor
VBIT	Villi binding inhibition test
V_H	Variable domain of a heavy chain
VHH	Variable domain of camelid heavy chain only antibody (Nanobody®)
VHH-IgA	fusion of the anti-F4 binding VHH to porcine IgA^b Fc
VHH-IgG	fusion of the anti-F4 binding VHH to porcine IgG3 Fc
V_L	Variable domain of a light chain
WHO	World Health Organisation

* Term coined in this thesis

Chapter 1

Scope and Objective

Developing an effective alternative to antibiotics
for prevention of post weaning diarrhoea in newly weaned piglets

One of the major burdens in the global pig-rearing industry is the disease called post-weaning diarrhoea (PWD), caused predominantly by enterotoxigenic Escherichia coli bearing F4 fimbriae (F4+ETEC) (Fairbrother et al., 2005). This disease causes reduction in weight gain and in extreme cases (~2% reported in Belgium) it leads to death of piglets. Consequently, both of these conditions lead to unavoidable economic losses for the farmers (Amezcua et al., 2002). In Belgium itself, ETEC related PWD leads to losses of about EUR 15 million every year, which is roughly about 1% of the annual returns of porcine meat, and a significant reduction of already low profit margin1. Globally, there are no solutions towards post-weaning diarrhoea caused by F4+ETEC. Antibiotics were used prophylactically until recently, but because of an increasing occurrence of antibiotic resistance, a ban on the prophylactic use of antibiotics was installed. However use of antibiotics is still prevalent, but now as therapeutic against PWD. The serious risk of introducing antibiotic resistant strains has led to a worldwide increasing demand for alternative strategies to prevent PWD. Some of these antibiotic alternatives include probiotic, prebiotic feed additives or even organic acids; but none of these products sufficiently reduce or prevent incidence of ETEC related PWD (Fairbrother et al., 2005; Roselli et al., 2005; Vondruskova et al., 2010).

Of several experimented techniques to prevent PWD, better results are obtained with passive immunisation strategies. Piglets are protected when fed feed containing plasma protein obtained from porcine slaughterhouses which is anticipated to contain anti-ETEC antibodies or when fed with specific anti-ETEC antibodies obtained from immunized hen eggs (Niewold *et al.*, 2007; Yokoyama *et al.*, 1992). However it is difficult to regulate the proportion of antibodies from batch to batch from both these sources. Moreover the anti-ETEC antibodies produced in eggs are expensive, while the use of slaughterhouse derived animal products in animal feed is discouraged due to important health and safety concerns.

[1] The archive of Dierengezondheidszorg Vlaanderen (Animal Health Care, Flanders) http://www.dierengezondheidszorg.be/ondersteuning/praktijk_advies_publikaties_varkens/autopsie%20varkens%202007.pdf

The crucial initial step in colonization of the host tissue occurs by attachment of the F4$^+$ETEC bacteria to the microvilli via the F4 fimbriae. The adhesive characteristic of F4 fimbriae has been shown to be exhibited by the polymerizing subunits- FaeG (Bakker *et al.*, 1992a; Bakker *et al.*, 1992b). We therefore opted to obstruct this initial infection step of F4$^+$ETEC colonizing the host tissue by oral administration of adhesion-blocking anti-FaeG antibodies to just-weaned piglets. For convenience of oral administration, and cost effective production we aimed at producing these anti-FaeG antibodies in seeds, so that they may directly be incorporated in the starter feed of weaned piglets.

Thus, our main objective was to develop a general approach to protect just-weaned piglets by oral feed based passive immunization during the critical period of three weeks after weaning when piglets are most susceptible to infection by ETEC. We chalked out a 4-part strategy with the aim to achieve this objective.

In the first step we intended to develop a robust antibody with high binding affinity, which can also be easily expressed and bulk produced. Hence rather than conventional monoclonal antibodies, or single chain-Fc fusion antibodies we decided to opt for variable domains of heavy chain antibodies (VHH) of camelids (Hamers-Casterman *et al.*, 1993). Specific VHHs were to be isolated by immunizing a lama with the purified FaeG adhesin. On selection of best anti-FaeG monomeric VHHs they would be cloned and characterised.

In the second step we planned to graft the anti-FaeG VHH on the Fc domains of IgG3 (Butler *et al.*, 2009) and IgA^b (Brown *et al.*, 1995) to generate bi- or tetravalent antibodies. The porcine IgG3 and the IgA^b are both suggested of being fairly resistant to proteases and this attribute makes them an ideal Fc for oral immunization (Brown *et al.*, 1995; Butler *et al.*, 2009). Also the fusion with the Fc domains should lead to dimerization and thus increase the avidity. Additionally by co-expression of porcine joining chain and porcine secretory component with VHH-IgA fusion molecule we sought to investigate the production of secretory IgA (SIgA). SIgA are tetravalent, robust and have high retention time at mucosal surfaces.

In the third step the anti-FaeG VHH fused to the Fc domains of IgG3 and IgA^{b} would be expressed in seeds. Seeds have the advantage to accumulate high levels of proteins, expressing antibodies in seeds allows their long-term storage at ambient temperature and does not require any specialised downstream processing. Further seeds of crop like soybean, pea, barley, etc. can be incorporated in the feed of young piglets. Since transformation of these crops is tedious and lengthy process, we choose the model plant *Arabidopsis thaliana* that can be effectively transformed using the fast and convenient method of floral dip transformation. Thus to obtain an initial proof of concept, both the formats of anti-ETEC antibodies would be introduced in Arabidopsis under the control of a seed specific promoter to produce the recombinant anti-FaeG antibodies in the Arabidopsis seeds. The anti-FaeG VHHs fused to the Fc domains of IgG3 and IgA^{b} could then be tested for their capacity to agglutinate F4^{+}ETEC bacterial cells and to inhibit adhesion of F4^{+}ETEC bacterial cells to microvilli of piglets *in vitro*.

If the seed made antibodies would be functional in the *in vitro* assays, then the fourth step shall consists of *in vivo* testing of the *in planta* produced anti-F4^{+}ETEC antibodies. To realise this, high expressing transgenic *Arabidopsis* plants can be scaled up, the harvested seeds could be used to produce a feed formulation. Such feed can then be fed to newly weaned piglets to evaluate its protective efficacy on challenging with an F4^{+}ETEC strain.

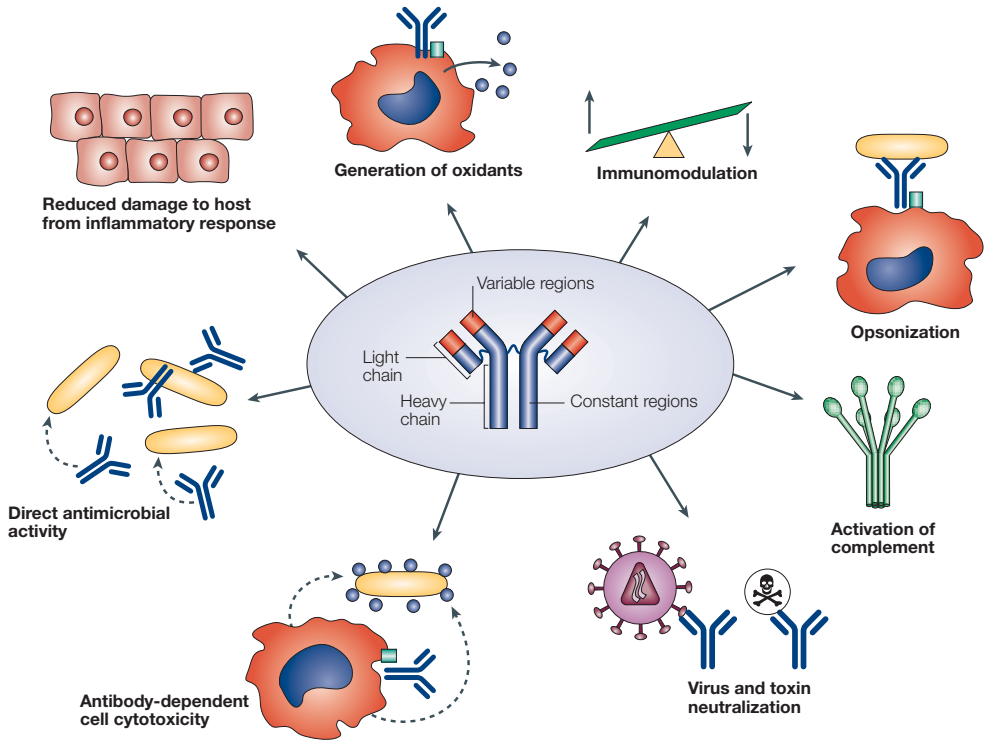
Chapter 2

Passive immunisation and the role of plant expression systems

A cost effective shortcut to attain protection against infection

Vikram Virdi, Sylvie De Buck, Henri De Greve and Ann Depicker

V.V. wrote the chapter and S.D.B, H.D.G and A.D edited it.



The above artwork depicts various biological functions of an antibody in evading pathogens, which can be achieved by direct administration of specific antibodies. Adapted from Casadevall et al., 2004

Abstract:

On encountering a pathogen, the immune system can develop specific resistance to control the spread of infection and prevent any subsequent infection of the same pathogen. The molecules called antibodies are a key factor in establishing this immunity. Vaccines are aimed at priming of the immune system and development of protective antibodies usually prior (weeks to months) to the contingency of encountering a pathogen. However there are many circumstances in which vaccines might be less suitable, particularly in situations where immediate immunity is needed. To achieve immediate immunity pathogen-specific antibodies can be administered; this medical procedure is called passive immunisation.

Passive immunisation has proven to be life saving in many acute infections, e.g. human respiratory syncytial viral infections in neonates, hepatitis B virus infection, etc. Apart from infectious diseases, passive immunisation is now also used within the cancer treatment. Such passive immunisation therapies are extremely expensive since it requires large amounts of specific antibodies that are produced in recombinant systems. Today most therapeutic antibodies are produced in mammalian cells, and attempts are being made to produce antibodies in cost effective alternative expression systems. Antibodies can be produced in plants, and since plant production systems require relatively less capital investments, the final cost of plant made antibody is estimated to be a fraction of the current cost. In addition, plant production systems are not prone to mammalian pathogens and hence provide a safe expression system. This eases the downstream processing and helps in maintaining the benefit of low production cost. The current molecular techniques in the field of *in planta* expression have enabled high-level expression of a variety of antibodies. These antibodies can be specifically produced in different plant organs like roots/tubers, leaves, seeds etc. of a variety of plants like potato, tobacco, maize, rice, pea etc. Providing for a very wide range of possibilities to develop a plant-based passive immunisation therapy, for instance production of antibodies in edible tissue allows for convenient needle-less oral passive immunisation at the gastric mucosal surface.

This review intends to shed light on the role of plants as a flexible expression system for passive immunotherapy. We build the discussion by revealing the scope of passive immunisation (i), then the various antibody formats that can help in achieving the goal of immune prophylaxis (ii), followed by a brief introduction to the current expression systems (iii) and then describe plant-based production system (iv), laying more emphasis on in leaf and in seed production for therapeutic application in humans as well as animals.

General introduction and scope of passive immunisation

One of the most important arsenals in fighting against infectious diseases is the protein molecule called immunoglobulin (Ig) or antibody (Ab). On natural infection and on vaccination, the immune system is primed to produce pathogen-specific antibodies, which in turn protect from subsequent infection by the same pathogen through various mechanisms (discussed later)(Durandy *et al.*, 2009). Vaccination has been one of the single most important medical procedures developed to attain long-term protection against disease-causing pathogens; it has enabled eradication of dreaded disease like small pox, and pushed polio near verge of extinction[2]. However, vaccination is predominantly prophylactic and usually needs to be administered well in advance to protect against possible infections. In many instances vaccines are not suitable, like protection of neonatal from vertical transmission of viruses from mother, protection against biological warfare, emerging diseases, protection of neonatal and pre-mature births, protection of elderly and immunocompromised patients; or in case of vaccine non-responders, or individuals at risk of vaccine-induced side effects. In most of these cases immediate protection is needed. In such instances immediate protection can be achieved by direct administration of disease-specific protective antibodies. The administration of disease-specific antibodies either prophylactically or post-exposure is called passive immunisation (Gonik, 2011; Naz and Rajesh, 2004; Raab, 2011; Zeitlin *et al.*, 2000; Zeitlin *et al.*, 1999). (Differences between active and passive immunisation: see table 2.1)

Passive immunisation occurs in nature as well, where it is called maternal immunity i.e. antibodies are transferred from mother to the offspring. In most

[2] http://www.cdc.gov/vaccines/vac-gen/downloads/pg_why_immz.pdf

animals, the transfer occurs through the placenta before birth (Garty *et al.*, 1994; Zhang *et al.*, 2012) and in mammals also later through maternal milk in early life (Hasselquist and Nilsson, 2009). In farm animals like pigs, horse, sheep and cow the transfer of maternal immunity occurs exclusively through colostrum and milk, as the placentation in these animals does not allow transfer of immunoglobulins (Hurley and Theil, 2011). In these economically important farm animals, the neonatal and newly weaned vulnerable young ones can be protected against many common diseases via artificial prolongation of passive immunity post weaning.

Table 2.1: Passive immunisation versus active immunisation

Points of distinction	Passive immunisation	Active immunisation
Provides immediate protection	Yes	No
Duration of protection	Days to months	Years
Systemic immunity	Yes	Yes
Capacity to restriction exclusively to mucosal surface if intended	Yes	No
Controlled duration of activity	Yes	No
Requirement of antigen specific host response	No	Yes
Diverse response with multi-specificities	Not with a single monoclonal antibody but possible with an oligoclonal mixture of different monoclonal antibodies or polyclonal antibodies	Yes
Prophylactic or post exposure	Both possible, ideally prophylactic or immediately on exposure (longer the duration of time after exposure, weaker is the protection)	Mostly prophylactic use (Except in case of few exceptions like rabies)

Table adapted from Zeitlin *et al.*, 2000 and Casadevall *et al.*, 2004.

The use of passive immunisation for prevention of infectious disease in human subjects can be traced back to the 1800s, when antibodies were sought from animal sera like rabbit and horse (Eibl, 2008). However, infusion of animal serum was not tolerated leading to immune complication called 'serum sickness' particularly on repeated use. Later human antibodies (from human convalescent

sera) were available and passive immunisation was largely used during the World War I, to treat tetanus, diphtheria and pneumococcal disease. By 1930s and 1940s, the advancement of fractionation techniques meant that the plasma proteins could be separated and a stable biological fraction could be administered. Such fractionated serum was used to treat poliomyelitis, mumps, measles, pertussis and even hepatitis A (Raab, 2011). Later, the use of passive immunisation declined with the emergence of antibiotics and vaccines (Berghman *et al.*, 2005; Hsu and Safdar, 2011) but passive immunisation is still in use (Holliger and Hudson, 2005; Naz and Rajesh, 2004). Moreover in recent years, due to increasing ban on antibiotics given the risk of introducing resistant bacterial strains, a suitable alternative is urgently needed. It is anticipated that passive immunisation will regain popularity and might reduce the dependency of the traditionally used antibiotic therapy (Berghman *et al.*, 2005; Oleksiewicz *et al.*, 2012; Zeitlin *et al.*, 2000). The technology for development of antibodies has developed in leaps and bounds, now specific high quality antibodies against one specific epitope- called monoclonal antibodies (mAb) can be produced (explained in detail in subsequent section). The technological developments, have thus give a boost to the passive immunisation field and aim to provide a part of the solution to the increased disease burden and emergence of new pathogens.

Along with infectious diseases (Table 2.2 and 2.4), passive immunisation is also being used to treat non-infectious diseases like– cancer (e.g. FDA approved, Bevacizumab and Cetuximab for colon cancer, Alemtuzumab for chronic lymphocytic leukaemia), autoimmune diseases (e.g. FDA approved, Adalimumab for treatment of rheumatoid arthritis) and Alzheimer (Bapineuzumab and Solanezumab are among some mAb being evaluated in clinical trials). For some more examples of various FDA approved clinically used antibodies, and other promising mAbs in clinical and preclinical phase of development see Table 2.2 and 2.4. (Casadevall *et al.*, 2004; Holliger and Hudson, 2005; Zeitlin *et al.*, 2000). Antibody therapy is also being used in novel applications like regulating and controlling drug abuse in case of cocaine and nicotine addiction. Antibodies specific for these drugs prevent their access to the brain, and thus regulate the drug induced effects. Currently ongoing clinical trials will provide more

information about this novel application of antibody therapy. For more on passive immunisation to treat drug abuse see review by (Kosten and Owens, 2005).

Mucosal surfaces comprising the respiratory tract, the gastrointestinal tract, the reproductive and the genital tract are the gateways for most pathogenic infections. Most vaccines fail to attain systemic as well as mucosal immune response, the latter can prevent the pathogenic invasion at the first port of entry. Topical application of antibodies at mucosal surface can immediately provide this barrier protection (Corthésy, 2003; Corthésy and Spertini, 1999). A mixture of 3 mAb 2G12, 2F5, 4E10 as a mAbGel is being evaluated in clinical trial as vaginal microbicide for prevention of HIV in heterosexual couples. Development of such microbicides against sexually transmitted diseases (STD) has profound importance in preventing unsafe sex-related death and disability (Whaley *et al.*, 2011).

Table 2.2: Examples of some promissing antibody and antibody derived fragments

Indication/pathogen	Brand name (generic name)	Antigen/specific target	Stage
Monoclonal			
RSV	Palivizumab	F protein	FDA approved
Anti-angiogenesis	Bevacizumab	VEGF-A	FDA approved
Rheumatoid arthritis	Actemra (Tocilizumab)		FDA approved
HIV	Tanox (Ibalizumab)	CD4	Phase IIb
Rabies	Foravirumab	Glycoprotein G	Phase II complete
Fab fragment			
Cardiovascular disease	ReoPro	GpIIb/GpIIa	FDA approved
Rattle snake bite	CroFab	Snake venom	FDA approved
Macular degeneration	Lucentis	VEGF	Phase III
ScFv			
Coronary artery bypass	Pexelizumab	Complement C5	Phase II/III
Tetravalent ScFv			
Melanoma	SGN-17	P97	Preclinical
ScFv dimer-Fc (minibody)			
Colorectal cancer	T88.66	CEA	Preclinical
Diabody			
Ovarian and breast cancer	C6.5K-A	Her2/Neu	Preclinical
VHH (camelid)[3]			
RSV	ALX-0171 (trivalent)		Phase I
Antithrombotic	ALX-0081, ALX-0681	Von Willebrand factor	Phase II

Compiled from Holliger and Hudson, 2005; Ter Meulen, 2011, FDA website

Passive immunisation or antibody therapy has great potential for human and animal health. The current cost of this therapy is often very high and a burden on

[3] http://www.ablynx.com/en/research-development/pipeline/

health care system (Rietveld *et al.*, 2010). Aiming at reducing the cost of production and treatment; different expression platforms are now being refined and several different antibody formats with engineered merits are being produced. In subsequent sections we introduced the various antibody and antibody-derived formats that have been developed and the current platforms used by industry to manufacture them, and lastly we explore how plants can provide a unique solution.

Antibody structure, formats, function and derivatives

The first Nobel Prize ever for Medicine was awarded in 1901 to Emil von Behring for the discovery of what was then called the 'serum antitoxins'. In 1890, Emil Behring and Shibasaburo Kitasato immunised rabbits with tetanus and then collected the serum; they noticed that when they administered 0.2 ml of this (immune) serum to healthy mice, the mice were protected from subsequent challenge from the virulent strain of tetanus; thus the concept of passive immunisation came in to being. Later (1939) Tiselius and Kabat discovered on electrophoreses of serum that the protective molecule within the serum– now know as 'antibody', belonged to the globulin fraction (hence called immunoglobulin). Using enzymatic and chemical methods the basic structure of the antibodies was deciphered. The antibodies are composed of two (long) heavy chains and two (short) light chains, which are arranged in a 'Y-shaped' structure (Figure 2.1, a).

The two (upper) ends of the 'Y' shaped molecule are called the 'Fab' region (fragment antigen binding) since they are involved in antigen binding. The variable tip domain of the paired heavy and light chains interacts with the antigen (labelled V_H and V_L in Figure 2.1). The tail end of the two paired heavy chains forms the 'Fc' (fragment crystallisable) region. The Fc region in a given class of antibody is conserved and attributes specific biological functions called effector functions. Depending upon the conserved domain of the heavy chain, the immunoglobulins can be classified as immunoglobulin G (IgG, bearing the γ heavy chain), immunoglobulin A (IgA, bearing α heavy chain), immunoglobulin D (IgD, bearing the δ heavy chain), immunoglobulin E (IgG, bearing the ε heavy chain) and immunoglobulin M (IgM, bearing the μ heavy chain). The IgG, IgD and

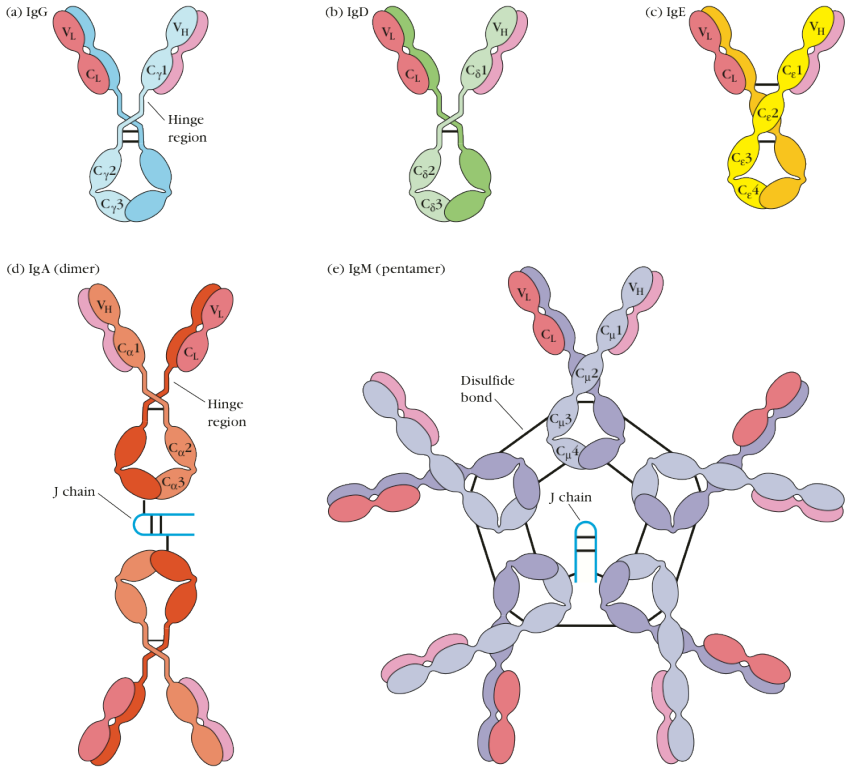


Figure 2.1: The 5 different antibody classes commonly found in mammals. The light chains are indicated in pink, each domain is shown as a connected oval, note the different number of constant domains in heavy chain. The disulphide bonds are indicated with a dark line. Here the IgA molecule is depicted as dimeric form joined by the Joining chain (blue – paper clip like), the pentameric IgM also bears a similar J chain. Reused from Goldsby *et al.*, 2003. For more details see text.

IgE are found in monomeric state in the serum (i.e. two heavy chains and two light chains — H_2L_2) (Figure 2.1, a, b and c) while the IgM occurs as a pentamer $(H_2L_2)_5$ (Figure 2.1, e). The IgA occurs as polymeric IgA (pIgA) either as tetrameric [$(H_2L_2)_4$ + J chain] or dimeric [$(H_2L_2)_2$+ J chain] form. A joining chain called 'J chain' facilitates the multimerisation of IgM and IgA (Figure 2.1, d and e)(Goldsby *et al.*, 2003). IgA is the predominant antibody at most mucosal surfaces where it occurs as secretory IgA (SIgA), i.e. a dimeric IgA (dIgA) with a secretory component wound around the complex (Corthésy, 2002; Strugnell and Wijburg, 2010)(please see Figure 5.1 in Chapter-5 for biogenesis of SIgA). The basic 5 antibody classes (IgG, IgA, IgM, IgE, IgD) can be found in mammals (Goldsby *et al.*, 2003). While some general differences are seen in other vertebrates e.g. except mammals only birds produce SIgA (Wieland *et al.*, 2004). In classes *Reptilia* and *Aves*, especially in the eggs of animals belonging to these two classes a variant class of Ig– the immunoglobulin Y (IgY, yolk antibodies) is

found (Chrzastek and Piasecki, 2011).

Besides these antibody formats, about two decades ago a unique kind of antibody occurring as a homo-dimer of heavy chains lacking light chains was discovered in camelids (camels and lama) and was named heavy chain antibody (HCAb) (Hamers-Casterman *et al.*, 1993). Soon after, similar heavy chain only antibodies were also discovered in cartilaginous fishes (nurse shark and wobbegong), this immunoglobulin was named Ig-NAR where NAR stands for 'novel antigen receptor' (Greenberg *et al.*, 1995). These two serendipitous discoveries gave an impetus to the development of the latest trend of engineered antibody with customised functions, for specific application (Muyldermans, 2001; Wesolowski *et al.*, 2009). The antigen-binding tip ends of these camel HCAbs are called the variable domains of heavy chain only antibody (VHH) or nicknamed Nanobody® while the variable domains of the shark Ig-NAR are called variable domain of novel antigen receptor (V-NAR). Both these antigen binding variable domains are highly evolved. The VHHs exhibit a long convex loop surface that increases the repertoire of antigen binding capacity. These VHHs and V-NARs have the ability to bind into deep enzyme clefts and thus target hidden conformational antigenic potentials, which are inaccessible to the conventional Igs (de Marco, 2011; Muyldermans, 2001; Muyldermans *et al.*, 2001).

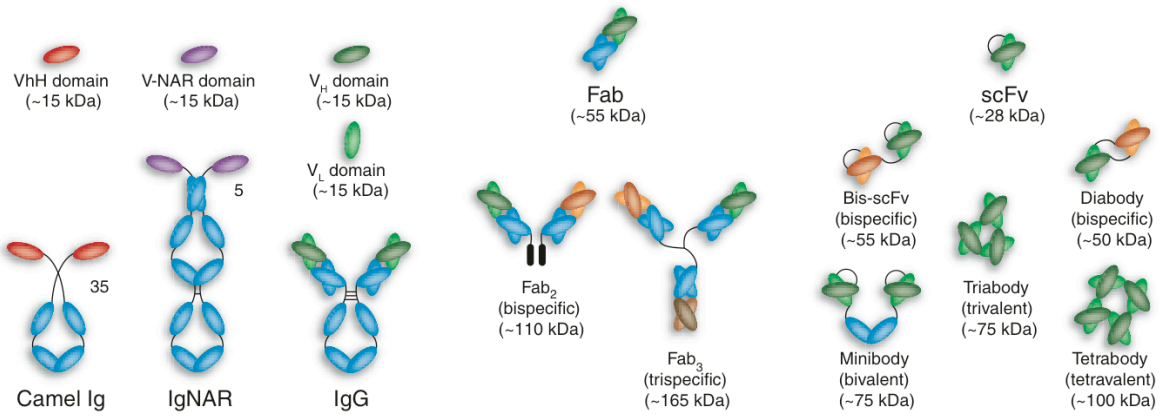


Figure 2.2: Schematic representation of full-length antibodies and various antibody fragments. The constant domains are indicated in blue. The classical IgG structure is shown along side the heavy chain only immunoglobulins from camelids (Camel Ig) and sharks (IgNAR); more explanation in text. The approximate molecular weight is indicated in brackets. The minibody and Fab_2 are bound by disulphide bonds within the hinge region, while the tri-specific Fab_3 is produced by chemically crosslinking (sulfhydryl-specific cross-linker o-phenylenedimaleimide) of 3 Fabs specific against different epitopes. The other variants based on scFv shown in the figure involve fusion through different size linker. Reused from Holliger and Hudson, 2005.

The wide range of these antibodies together with the state of the art chemical and genetic engineering tools, together with the choice of host expression systems have now presented the researchers with a myriad of possibilities to construct customised

antibody / antibody-like molecules. This can be comparable to a Lego® kit where the synthesis of novel antibodies is constrained merely by human imagination (Carter, 2006; Holliger and Hudson, 2005; Saerens *et al.*, 2008) (see Figure 2.2).

The construction of these new antibody derivatives are predominantly governed by the application demand, for instance by joining the variable domains of the heavy and light chain (V_L + V_H) a minimal sized, antigen binding single chain variable fragment (ScFv) (Figure 2.2) can be made. Further by linker and fusing this assembly to the hinge and Fc leads to the construction of single chain antibodies or ScFv-Fc (Figure 2.2). The ScFv-Fc is a molecule which is lighter so has the attributes of higher penetration but at the same time is capable of elicitation all the effector functions of the Fc, like cytotoxic effect, interaction through Fcγ receptor, increased half life through interaction with the neonatal Fc receptor (FcRn) etc. (Holliger and Hudson, 2005). The other advantage of ScFv-Fc is the ease in expression as just one transgene is needed rather than co-expression of two transgenes (encoding the light and heavy chains) as in case of mAb (Schirrmann *et al.*, 2008; Xu *et al.*, 2011a).

Alternatively, if not needed for a specific function, the Fc of a ScFv-Fc or of a mAb can be relegated and just the antigen binding domains can be produced (Fab or ScFv), especially for instances where even higher degree of penetration is needed like in case of delivery of carrier molecule (for imaging or chemo-toxin) into tumour cells. In such application the faster renal clearance of a small molecule is also beneficial (both for imaging as it enables reduction of background, and in target drug/toxin delivery)(Holliger and Hudson, 2005) (Table 2.2 and Figure 2.2).

Antibodies with higher affinity are much desired for *in vivo* administration. By making the antigen binding domains multivalent an enhanced functional affinity can be achieved, this is termed as avidity (as naturally seen in pentameric IgM, di- and tetrameric IgA). There have been several examples where using Fab, ScFv or VHH/V-NAR bivalent, trivalent and tetravalent antibody-like molecules have been produced and some are being evaluated in clinical trials (Figure 2.2, Table 2.2). Further by fusion of antibody fragments recognising different epitopes bispecific and trispecific antibodies have been produced which present with a

unique potential to prevent pathogenic infections. For more details on the fascinating plethora of antibody derivatives see the reviews by Holliger and Hudson, 2005; Saerens *et al.*, 2008; Wesolowski *et al.*, 2009.

These varied formats of antibodies have been expressed in several different host systems; of these some are making their way to the market while several other perspective antibody formats are in clinical trial. Table 2.2 presents some examples of these promising antibody and antibody fragments.

Table 2.3: Different mechanisms by which antibodies mediate protection against specific pathogens

Mechanism	**Examples**
Agglutination	Crosslinking of bacteria like *Vibrio cholera* (Corthésy and Spertini, 1999) and *Streptococcus mutans* causing dental carries (Ma *et al.*, 1990) is important for protection.
Trapping in mucus	Specific antibodies help in entrapment of larvae, nematodes, bacteria which is followed by rapid expulsion (Corthésy, 2002)
Inhibition of pathogen target cell interaction by preventing attachment or fusion or internalisation	– Neutralising activity of polyclonal IgM in case of influenza virus in predominantly due to preventing attachment to target cells – Anti-herpes simplex virus monoclonal antibodies (mAb) prevents fusion *in vitro*, and this mechanism is suggested to protect from vaginal challenge – Anti-reovirus mAb prevents uncoating and internalisation of the virus
Neutralisation of toxin	Toxin-specific antiserum protects against *Staphylococcus aureus* infection Antidote to snake bite venom is a cocktail of animal derived anti-venom immune sera (see WHO snake antivenom website– http://goo.gl/Js03h)
Complement activation	Protection offered by anti-RSV mAb failed on destroying the complement system with cobra venom factor (CoVF) (Corbeil *et al.*, 1996)
Antibody-Dependant Cell-mediated Cytotoxicity (ADCC) of infected cells	The action of malaria immune sera used in human therapy is associated with ADCC activity and not in inhibition of infection
Phagocytosis via Fc receptor	Anti-*Pseudomonas aeruginosa* antibody enhances phagocytic activity and increases survival of challenged guinea pigs

Table adapted from Zeitlin *et al.*, 2000, references only to additional examples are cited. Also see recent review by Durandy *et al.*, 2009.

Antibody production platforms

There has been surging interest in the manufacturing of recombinant monoclonal antibodies and antibody fragments. It is estimated that antibody production business accounts for about US$30 billion[4], which is likely more than 30% of the total capital earnings from biotech industry (Holliger and Hudson, 2005). Given the large demand for the antibody and antibody fragments, several different production systems have been developed to meet this requirement and deliver high quality products (Schirrmann *et al.*, 2008). Production of monoclonal antibodies has been made possible with the invention of hybridoma technology, this involves fusion of spleen cells from an immunised mouse (or rat) with immortalised myeloma cells (Kohler and Milstein, 1975). Today, this platform contributes to some of the major demand of monoclonal antibodies in research and diagnostics. However for therapeutic application, the use of murine IgG is discouraged due to possible side effects arising from immune response to foreign IgGs. Hence for human application humanised or at least partially humanised antibodies are needed. The production of human hybridomas has been difficult; instead B cells from transgenic mice with human IgG gene repertoire are used to generate humanised immunoglobulin (Bruggemann *et al.*, 1989; Schirrmann *et al.*, 2008). There are drawbacks of hybridoma technology for passive immunisation as it is inefficient in producing antibodies against toxins and conserved antigens, more over it requires immunisation and relies on development of an *in vivo* immune response (Schirrmann *et al.*, 2008). *In vitro* technologies like phage display have liberated from these constrains, and now gene element of the antigen binding antibody domains can be quickly sequenced (Smith, 1985). Also in recent years, the *in vitro* technology for development of human monoclonal directly from a single B cell derived from an ampule of peripheral blood mononucleocytes is well established (Tiller *et al.*, 2008). This is done by sequestering the specific antibody coding genes using state of the art molecular biology tools. Further these gene elements can then be optimised for expression in various systems. This technique has greatly contributed to discovery and development of antibodies particularly for HIV neutralising antibodies from infected donors (Andrabi *et al.*, 2012).

[4] http://www.alderbio.com/5/MARKET%20POTENTIAL/

Bacterial expression systems are very popular for expression of various enzymes and proteins of commercial interest. Both Gram-negative and Gram-positive strains have been explored for production of antibodies and antibody fragments (Schirrmann *et al.*, 2008). However, one of the most important prerequisite for correct antibody assembly is the targeting to an oxidising subcellular environment and precise glycosylation (Better *et al.*, 1988). Expression in the reducing environment of the prokaryotic cytoplasm usually results in improper folded antibodies usually forming aggregates within the inclusion bodies (Philibert *et al.*, 2007; Schmidt, 2004). Targeting to the periplasmic space (oxidising environment) has been successful for the formation of disulphide bonds, and thus facilitating functionality of antibody and antibody fragments (Sletta *et al.*, 2007). While in case of Gram-positive bacteria direct secretion into the medium has been tested as well. The expression in the periplasm has been commercially feasible mostly for production of antibody fragments like Fabs and VHHs, and not so much for production of full length Ig (Schirrmann *et al.*, 2008). Expression of full length IgG (aglycosylated) though difficult, can be produced by fine tuning the regulatory sequences and optimal periplasmic secretion (Simmons *et al.*, 2002). The other demerits of the bacterial expression system are plasmid loss, mutations in the vector, and low yields in some cases due to toxicity of the host. However in optimised condition, high expression of antibody derived fragments can be achieved, e.g. the anti-hapten ScFv was reported to express up to 1.2 g/L in a bioreactor (Sletta *et al.*, 2004).

Eukaryotic systems are preferred in many a case, especially for the advantage of folding, assembling and precisely attributing posttranslational modifications; for instance the glycosylation of Fc which is required for its effector functionality (Chowdhury and Wu, 2005). Today– yeast, filamentous fungi, insect cells, mammalian cells, plant cells, and even transgenic animals and plants are being used for the production of antibody and antibody fragments for therapeutic use. Each of these systems has its own merit and reasoning for development to enable large scale production of a specific Ig like molecule (Schirrmann *et al.*, 2008). Yeast systems have the advantage of being able to scale up in large bioreactors; it does not produce endotoxins, which is advantageous for downstream processing in therapeutic use. More over the antibody/antibody fragments can be secreted

Table 2.4 Recent developments in antibacterial antibodies for clinical passive immunisation

Bacteria / indication	Antibody	Target	Origin / antibody class	Remark
Botulinum toxin (*Clostridium botulinum* toxin)	XOMA 3AB (equimolar cocktail of three NX01, NX02 and NX11 mAbs)	Targeting different epitopes on the serotype A, coverage extending to subtypes A1, A2, A3 and A4	Humanized monoclonal antibodies (IgG1)	Phase I
Shiga toxin-producing *E. coli* (STEC)	Shigamabs (Thallion Pharmaceuticals, mAbs cαStx1 and cαStx2)	Cocktail of two humanized antibodies against Stx1 and Stx2 toxins	Humanized (IgG1)	Phase II (Granted orphan drug status in US and EU)
Clostridium difficile	GS-CDA1 (Massachusetts Biologic Laboratories /Medarex/Merck)	Toxin A	Human mAb	Phase II
	MDX-1388 (Massachusetts Biologic Laboratories /Medarex/Merck)	Toxin B	Human mAb	Phase III
Anti-sepsis *E.coli* endotoxin (lipid A)	Nebacumab (Centoxin or HA-1A, Centor)		IgM, from splenocytes of a Hodgkin disease patient vaccinated with heat-killed rough *E. coli* J5	Safety concerns & failed efficacy
	edabacomab (Xomen-E5, XOMA)		IgM, From splenocytes of BALB/c mice immunized with heat-killed rough *E. coli* J5	Failed efficacy
	T88 (Chiron)		IgM, from non-immunized volunteer	Failed efficacy
Lipoteichoic acid of Gram-positive bacteria	Pagibaximab (BSYX-A110, Biosynexus and GSK)	lipoteichoic acid, a highly conserved surface component of *S. aureus* and coagulase-negative *Staphylococci*	Chimeric mouse/human IgG1 antibody,	Phase III (prevention of Staphylococcal sepsis in low weight infants)
Staphylococcus aureus	Tefibazumab (T1-2 or Aurexis, Inhibitex)	Clumping factor A (ClfA) of *S. aureus*	Humanized IgG1	Phase II trials 2005–2006 (Ineffective)
	Veronate (INH-A21 Aurexis)	ClfA of *S. aureus* (and SdrG of S. *Epidermidis)*	High titer Humanized IgG preparation	Phase III (Failed efficacy)
	Aurograb (Developed by Neutec, licensed by Novartis)	GrfA surface protein of S. aureus	scFV selected from a human scFv phage library	Synergistic effect with vancomycin, entered phase III in 2004–2006 (failed)
	F958 (Developed by Alopexx Pharmaceuticals, licensed by Sanofi–Pasteur in 2010)	Poly-N-acetylglucosamine (PNAG), a surface polysaccharide of *S. aureus*	Human mAb	Preclinical
Pseudomonas aeruginosa	KB-001 (KaloBios Pharmaceuticals)	Targets the conserved PcrV protein	PEGylated Fab (from mAb 166)	Placebo-controlled phase I/II trial in cystic fibrosis patients was tolerable, safe and non-immunogenic (2010). Placebo-controlled phase I/II trial in France with 36 ventilated patients-reduced the occurrence of pneumonia (31–33% vs 60% in placebo) (2010)
	Panobacumab (KBPA-101, Kenta Biotech)	O-antigen carbohydrate of serotype O11	Immunizing human volunteers	Effective in phase II a (Exclusively specific to O11 strain)
	Aerucin (Aridis Pharmaceuticals)	Specific to the surface polysaccharide alginate.	Fully human IgG1	Preclinical
Anthrax protective antigen (PA)	Raxibacumab (Human Genome Sciences)	PA domain IV	Human antibody from human phage display library	Protective in preclinical studies with primates
	AVP-21D9 (Avanir Pharmaceuticals)	PA domain III	From human peripheral blood lymphocytes	Interferes with toxin assembly, effective in preclinical studies in rabbits
	Anthim (Elusys Therapeutics, ETI-204)	PA domain IV	Humanized mouse mAb 14B7	Preclinical efficacy demonstrated in rabbits
	Valortin (PharmAthene/Medarex, MDX1303)	PA domain III	Human mAb from HuMab transgenic mice	Preclinical protection in rabbits and monkeys on lethal challenge of spores

Table has been summarised from recent review by Oleksiewicz *et al.,* 2012 and the referenced mentioned there in. (More details can be found on the company websites mentioned in parenthesis. Tip: see clinicaltrial.gov for updates of the clinical trial.)

out of the host cells. The secretory proteome of commonly used yeast strains for recombinant protein production like *Pichia pastoris* and *Saccharomyces cerevisiae* is also very simple, which eases purification (Fischer *et al.*, 1999).

In *P. pastoris* the expression of ScFv has been reported as high as 1.2 g/L under optimised conditions (Freyre *et al.*, 2000), while *S. cerevisiae* can yield over 100 mg/L of lama derived VHH (Frenken *et al.*, 2000). Fab and IgGs can also be produced in yeast by co-transformation of both the antibody chains fused to the alpha-factor prepropeptide leader sequence. Some of the initial attempts with this co-transformation technique yielded 1 to 50 mg/L assembled molecules in flask culture which when scaled up to bioreactors gave up to 0.5 g/L (Horwitz *et al.*, 1988). However functional full length Igs were seldom produced in yeast cells. Traditional difficulties with yeast expression included the lower transformation efficiency, failure in expressing AT rich regions, proteolysis of the secreted proteins in high-density cultures, and hyper-glycosylation (Schirrmann *et al.*, 2008; Sethuraman and Stadheim, 2006). Latest developments in the field, including methods like synthetic gene optimisation and strain modification are providing impetus to the use of yeast for full-length antibody production. For example, Potgieter and co-workers have developed a glycoengineered *P. pastoris* strain, that is reported to yield more than 1g/L of functional monoclonal antibodies that bear uniform N-link glycans (Potgieter *et al.*, 2009). Such modified strains provide for robust, scalable platform that might rival mammalian production systems.

Mammalian cell cultures have been readily better suited for obtaining highly functional proteins with mammalian glycosylation. It is due to this reason that the majority of therapeutic antibodies administered in clinics are produced in mammalian cells despite the high cost of production. The yield of the antibodies produced in mammalian cells has increased more than 10 folds since 1980s to more than 5 g/L (Wurm, 2004). This high production level has been possible by achieving high cell density, high antibody expression per cell and improvement in chromosomal integration of antibody producing genes. However stable transformation of mammalian cells is a lengthy process. As an alternative, transient expression with viral promoters has empowered production of

hundreds of milligrams of antibodies (Schirrmann *et al*., 2008). Nevertheless the mammalian system is still expensive and has become the limiting factor for wide spread passive immunisation. Expression in transgenic plants and animals is a solution to effective scale up therapeutic Ig. Generation of transgenic animals like cows producing monoclonal antibody in milk is an exciting concept but is time consuming (Houdebine, 2009; Houdebine, 2002). In comparison, development of transgenic plants is rather easy. This relative ease of transformation and the recent development in technology for plant transformation tools has enabled effective production of almost all kinds of antibody and antibody formats (Paul and Ma, 2011; Xu *et al.*, 2011a), even with engineered human like glycosylation (Webster and Thomas, 2012). In the next section we describe the plant as antibody production platform in detail.

Table 2.5: comparison of different systems for antibody production

Features	**Bacteria**	**Yeast**	**Plant cell culture**	**Mammalian cell culture**	**Transgenic animals**	**Transgenic plants**
Production cost	Low	Medium	Medium	High	High	Low
Production timescale	Short	Medium	Medium	Long	Very long	Short, medium, long*
Propagation	Very easy	Very easy	Easy	Medium	Medium	Very easy
General Quality	Low	Medium	High	High	High	High
General Safety	Low	High	High	Low	Low	High
Ease of modification of glycosylation	Very difficult	Medium	Difficult	Difficult	Very difficult	Difficult

Table reused from Ko and Koprowski, 2005, * depends on the plant and the system, e.g transient *Nicotiana* expression platforms is one of the fastest production system, stable expression in Arabidopsis is not very lengthy, however antibody production in crop seeds like pea is time consuming- however scale up of bulk antibody is fast.

Plants as antibody production platform

The first proof of expression of functional antibodies in plants was provided in 1989 (Hiatt *et al.*, 1989). In the pioneering paper two transgenic tobacco plants each expressing light and heavy chain were produced by Agrobacterium-mediated transformation of tobacco leaf discs. Crossing these two transgenic tobacco lines led to the expression of assembled functional IgG antibodies, accumulating up to 1.3% of total soluble protein (TSP). From then on, numerous antibodies and other proteins have been expressed in plants, demonstrating that plants can express components of complex multimeric proteins, assemble these

polypeptides and produce functional proteins (De Muynck *et al.*, 2010; Hiatt *et al.*, 1989; Rybicki, 2010). Plants bear chaperons like BiP (binding protein) which facilitate the proper folding of Ig (Nuttall *et al.*, 2002).

Since 1989, there has been tremendous development in the expression of antibodies in plants (recently reviewed in De Muynck *et al.*, 2010; Obembe *et al.*, 2011; Paul and Ma, 2011; Whaley *et al.*, 2011; Xu *et al.*, 2011a). Plant expression systems are a very attractive platform for the production of antibodies, predominantly due to the possibility of up scaling large quantities of antibodies at a fraction of the cost as compared to conventional systems. It has been estimated that if the antibodies expresses up to 1% of TSP in plants, then the cost of production would be 0.1% of the mammalian cell culture system and up to 2-10% of the cost of production via microbial systems (Chen *et al.*, 2005). The other advantage is that plants do not harbour any of the mammalian viruses and other mammalian pathogens, making it comparatively safer expression system (Pogue *et al.*, 2010). This also adds to the convenience of purification and downstream processing of plant made antibodies, which ensures the low cost of the final product. Such purified plant made antibody can be applied parenterally or a semi-purified/crude preparation can be applied topically. Expression in edible plant tissues like tuber, roots, and seeds allows for oral delivery of antibodies. Oral delivery of plant made antibodies can allow for passive immunisation against enteric diseases, and is more advantageous in passive immunisation of farm animals. It is anticipated that the in seed/ in feed production will open a new avenue for passive immunisation of farm animals, which so far has been impossible due to the cost involved in passive immunisation using antibodies produced in conventional systems (Floss *et al.*, 2007). Additionally in seed expression, bears the benefit of storage at ambient temperature and ease of transportation, which can enable decoupling of the scale up (fields), and downstream processing (Khan *et al.*, 2012).

Different plant based expression systems

There is a huge diversity of green plants species found on our planet, which grow and flourish in different habitats right from aquatic unicellular plants like microalgae, multicellular aquatic flowering plants like duckweeds to land plants

like tobacco, Arabidopsis, etc. The drive to achieve abundant overall production with lower capital investment have led to exploration of all these different plant systems for the production of heterologous proteins (Xu *et al.*, 2011a). The common unifying element in these varied species is that all of these autotrophic plants have a relatively similar cellular machinery, glycosylation pattern, and most can be transformed by Agrobacterium-mediated transformation or by particle bombardment protocols (Ko and Koprowski, 2005; Xu *et al.*, 2011a). The Agrobacterium-mediated transformation is preferred whenever possible over particle bombardment, as it tends to introduce low copy number of the T-DNA in the plant genome (Cheng *et al.*, 2004; Ko and Koprowski, 2005). Via both these methods, stable plants expressing antibodies can be produced and homozygous lines can be identified for future bulk production of antibodies. Stable expression, identification of primary transformants, selection of best plant lines does take time, but once the primary seed bank is selected, future scale up can be done with existing farming infrastructure. Alternatively to stable expression (integration into the genome) and development of transgenic plant lines, one can opt for quick protein production by transient expression either by *Agrobacterium* infiltration (Kapila *et al.*, 1997) or by using viral vector expression system like the MagnIcon™ system (Icon genetics) (Gleba *et al.*, 2005) and Geminiviral expression system (Arizona State University) (Mason *et al.*, 2002) (Chung *et al.*, 2006). Each of these methods has its own merit, and depending on the antibody/antibody-fragment, the downstream processes, delivery system etc. the apt plant expression system can be chosen. Using these methods (stable and transient) antibodies have been produced in a variety of species and systems– like moss (Decker and Reski, 2008), microalgae (Franklin and Mayfield, 2005; Mayfield and Franklin, 2005), duckweed (Cox *et al.*, 2006), plant cell (Hellwig *et al.*, 2004), organ cultures (Hellwig *et al.*, 2004; Sharp and Doran, 2001) and in land plants– both in monocots (like maize) and dicots (like Arabidopsis, tobacco, potato, soybean, alfalfa etc.) (De Muynck *et al.*, 2010).

However, given the quality and quantity of the recombinant antibody that can be produced, currently leaves followed by plant culture system (hairy roots, cell culture, etc.) and seed tissue are most widely used (De Muynck *et al.*, 2010). Here we review the whole leaf and seeds expression system. For more information of

plant culture system see recent comprehensive review (Xu *et al.*, 2011b).

In leaves expression of antibody

The most important motivation for production of antibodies in leaves is the capacity to scale up high amount of biomass in multiple harvests per annum. De Muynck *et al.* published a survey of plant made full-length antibodies in 2010. According to this review more that 50% of plant made full length antibodies till then were expressed in leaves (De Muynck *et al.*, 2010). Of the plant species that have been explored for in leaf production of antibody and antibody fragments, seemingly tobacco (mostly *Nicotiana tabacum* and *Nicotiana benthamiana* species) has emerged as one of the leading platforms (Paul and Ma, 2011). Tobacco can yield up to 300 tons of biomass per acre, can be conveniently grown in fields as well as in greenhouses, different species have been well studied, and tobacco is not regarded as a feed/food plant; these merits make tobacco a very interesting plant for production of antibodies (and other high value recombinant proteins) (Xu *et al.*, 2011a). More importantly, genetic manipulations of tobacco are easy, and antibody genes can be introduced both stably in the nucleus via Agrobacterium-mediated transformation or can be transiently introduced by agro-infiltration, or by viral vectors (Whaley *et al*., 2011; Xu *et al*., 2011).

One of the most successful examples of tobacco made antibody for passive prophylaxis is the secretory antibody CaroRX™ from the company– Planet Biotechnology. This antibody is now available within the European Union for prevention of dental caries. SIgA antibodies are ideal for immunoprophylaxis at the mucosal surfaces, however recombinant production of these hetero-decameric SIgA antibodies is not commercially feasible by conventional production systems; and to date 'plants remain the only viable platform for production of SIgA antibodies' (Paul and Ma, 2011). All the elements needed for the expression of these SIgA are brought to one plant line by successive crossing of tobacco lines expressing light chain and heavy chain, then J chain followed by SC expressing line (Ma *et al.*, 1995; Wycoff, 2005). The ensemble of stable nuclear transformation and classical breeding technique has proven to be a robust system for production of such complex antibodies (Paul and Ma, 2011). Based on the same strategy Planet Biotechnology Inc. has developed another 2

promising products[5]. One of which is DoxoRX™ for drug induced alopecia, a common side effects of cancer therapy, while the other is a SIgA antibody like adhesion molecule RhinoRX™, where the typical antigen binding domains of SIgA are replaced by the rhinovirus binding cellular ICAM1 receptor (patent no. EP 1290027 B1). This tetravalent SIgA-ICAM1 assembly was 200 times more effective than soluble ICAM1 in impeding rhinovirus *in vitro*. It is anticipated that the multivalent SIgA-ICAM1 would have a longer life time in nasal mucosal application (Wycoff, 2005). The phase I trial of both these molecules is completed (Obembe *et al.*, 2011).

Another successful example of stable expression of antibody in tobacco leaves is the 2G12 anti-HIV antibody. This antibody was isolated from human sera and has drawn attention due to its characteristic property to neutralise various isolates of HIV virus (Paul and Ma, 2011; Trkola *et al.*, 1996). Within the pharma-planta consortium this antibody was expressed under the control of a constitutive promoter and tissue-specific promoters in seeds of maize (Rademacher *et al.*, 2008; Ramessar *et al.*, 2008) and Arabidopsis (Loos *et al.*, 2011a), and in the leaves of tobacco. The antibody produced in tobacco plant lines was chosen for further clinical evaluation, as a topical vaginal microbicide to prevent HIV transmission. The Phase I clinical trial (clinical trial no. NCT01403792) with purified 2G12 produced in tobacco leaves has been completed recently. The 2G12 antibody producing tobacco plants were grown in the contained greenhouse in compliance to cGMP (current Good Manufacturing Practices) procedures at Fraunhofer IME, Aachen, Germany (Paul *et al.*, 2011), and immediately purified on site by a customised downstream protocol. Growing of plants in regulated greenhouse condition is advantageous as it prevents the chances of gene flow to the environment through escape of pollen and dispersal of seeds. The regulated environmental conditions within the greenhouse like temperature, humidity, etc. also enable maintenance of quality and quantity from one batch to other, as it has been noted that biotic and abiotic factors do affect yield of plant made recombinant protein (Paul and Ma, 2011). Production in greenhouse increases the cost of the antibody being produced as compared to

[5] www.planetbiotechnology.com

field grown therapeutic antibody, but on the positive side it will pave way for establishing regulatory procedures (Paul and Ma, 2011; Paul *et al.*, 2011).

Exploring the advantage of contained growth condition, there are several excellent transient expression systems developed that enable large-scale production of recombinant protein including antibodies. Using these systems therapeutic proteins and antibodies can be produced in about 8 days after cloning of the target gene DNA. Some of these include systems like virus based expression systems magnICON (Gleba *et al.*, 2005), Gemini (Huang *et al.*, 2010; Mason *et al.*, 2002), Geneware (Pogue *et al.*, 2010), and engineered vectors for agro-infiltration like the pEAQ system, in which the T-DNA also bears the p19 viral silencing suppressor (Sainsbury *et al.*, 2010; Sainsbury *et al.*, 2009; Voinnet *et al.*, 2003). The possibility of high scale antibody production in limited space in greenhouses, over a short period of time enables multiple rounds of production. This also eases the downstream processing and enables high recovery of purified antibody. Companies and institutions like Medicago (Quebec city, Quebec, Canada), Kentucky BioProcesses (Owensboro, Kentucky, USA), Texas A&M (college station, Texas, USA), Fraunhofer (Newark, USA) and Icon Genetics (Bayer, Halle, Germany) have established infrastructure for large scale automated systems to grow tobacco plants in the greenhouse, infiltrate/infect, and harvest the protein (Whaley *et al.*, 2011). In our opinion one of the break-through possibilities budding from this technology platform would be for the sector of personalised antibody therapeutic. Icon Genetics, a subsidiary of Bayer (Germany), is developing patient-specific antibodies as idiotype vaccine to treat Non-Hodgkin's lymphomas (NHL). Cost effectiveness of this tobacco production system should make personalised cancer therapy accessible to larger number of patients. Currently the facility can produce antibodies up to 0.5 to 4.8 g/Kg fresh weight, while approximately 225 mg antibody per patient would be required for treatment as well as quality assurance tests. Once the antibody genes are isolated and cloned, from then onward in 2 weeks the antibody can be administer to the patient (Paul and Ma, 2011; Whaley *et al.*, 2011). The phase I clinical trial with tobacco made anti-idiotype vaccine for NHL is completed (McCormick *et al.*, 2008) and these transiently produced antibodies are now in clinical trial. The cost of bringing a product to Phase I clinical trial with cGMP mammalian system

is about $5-6 million, while in case of transient plant systems is about $0.5-0.8 million (Whaley *et al.*, 2011).

The quick transient production platform is also well suited for production of antibodies (or even vaccines) for pressing emergency situations like biological warfare. A laboratory proof of concept for production and efficacy of anti-Ebola monoclonal antibodies has been provided in the mice model (Wilson *et al.*, 2000). A primate study will soon start with the bulk-produced antibody in cGMP tobacco manufacturing facility (Whaley *et al.*, 2011). Similar projects aimed at immediate response with high manufacturing capacity at low cost for biological threats are being developed for Marburg virus, Lujo virus, *Staphylococcal* enterotoxin B, ricin etc. [6](Whaley *et al.*, 2011). Along with companies, government defence institution like DARPA (Defence Advance Research Project Agency of USA) have invested in the contract cGMP tobacco production platforms. It is aimed at producing three million dose of about 400 mg individual antibody dose, in within 1-3 months at a cost of less than US$10 per dose.

Some of the interesting antibodies for human therapeutic to watch out for in near future would be the anti-human respiratory syncytial virus (HRSV) mAbs. Currently passive immunisation with anti-HRSV antibodies (like Synagis®, Motavizumab®, Palivizumab®) is a routine treatment but is limited to patients with extremely serious condition, as the treatment is very expensive and unaffordable for many health systems in developing countries and burdens even the developed health care systems in many western countries with high per capita income (Harkensee *et al.*, 2006; Rietveld *et al.*, 2010). Tobacco production system is also being used for the development of antibody therapy for non-infections disease along with cancer, autoimmune disease and Alzheimer disease (for more details see review (Whaley *et al.*, 2011)).

In all, the tobacco leaf based expression both stable as well as transient seems very promising. On the negative side though, agro-infiltration does lead to abundant bacterial cells, introducing the risk of bacterial endotoxin contamination. And the leaves have to be processed immediately for optimal product recovery (Paul and Ma, 2011). However, innovative, cost effective

[6] http://www.mappbio.com/productdevelopment.html

purification systems like the tobamovirus–protein A fusion are being developed (Werner *et al.*, 2006); incorporating these inventions shall maintain the cost effectiveness of plant expression systems.

In seed antibody expression

Seeds can be regarded as natural protein production factory and a storage house. As, in addition to the merits of *in planta* expression, in seed expression provides for long term storage, ease of handling and transport without cold chain maintenance and it can double up as an oral delivery system as well (Floss *et al.*, 2007; Khan *et al.*, 2012). Thus seed can be used directly for passive mucosal immunisation, which is particularly advantageous for animal disease (Floss *et al.*, 2007; Zimmermann *et al.*, 2009) and also for human application. Alternatively, for parenteral application, the high concentration of protein in desiccated seeds facilitates the downstream processing. Different full-length antibody and antibody formats like ScFv, ScFv-Fc, VHHs, VHH-Fc have been expressed effectively in seeds of both monocot and dicot plants (De Wilde, 2012; Khan *et al.*, 2012; Loos *et al.*, 2011b; Van Droogenbroeck *et al.*, 2009) (Table 2.7).

Brief introductions to seeds

Anatomically seeds have three important parts, the outer protective seed coat, enclosing the endosperm and the embryo. In angiosperms the characteristic double fertilisation event leads to the production of the embryo and endosperm, which is the next plant generation and the nutritive tissue respectively. The embryo in these flowering plants can comprise of a single cotyledon, their seeds are hence called monocotyledonous (Figure 2.3, d) or they might bear two cotyledons then the seeds are called dicotyledonous (Figure 2.3, a, b and c). Further in some dicotyledonous plants the endosperm might be reabsorbed during seed development, such seeds are classified as non-endospermic dicotyledonous seeds as oppose to endosperm bearing endospermic dicotyledonous seeds. Leguminous plants like pea (*Pisum sativum*) (Figure 2.3, b), garden bean (*Phaseolus vulgaris*), soybean (*Glycine max*) and most species of the family Brassicaceae like rape (*Brassica napus*), wild mustard (*Sinapis alba*), wild radish (*Raphanus sativus*) are examples of non-endospermic dicotyledonous seeds. The endospermic dicotyledonous seeds on the other hand might have a

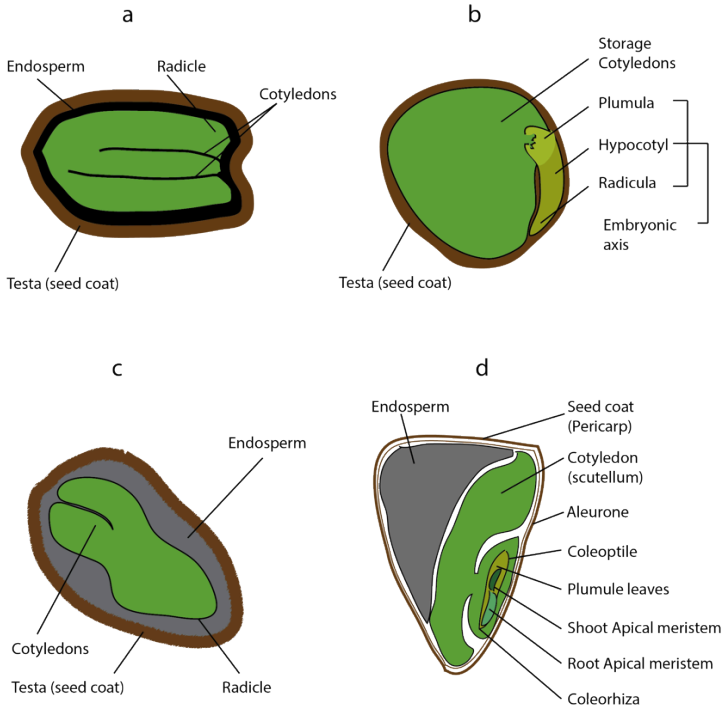


Figure 2.3: Classification of seeds based on basic seed anatomy. Angiosperm seeds are basically classified as dicotyledonous seeds (a, b, c) with two cotyledons and monocotyledonous seeds (d) with one cotyledon. The dicotyledonous seed can be non-endospermic, lacking endosperm completely e.g. peas (b); or they can be endospermic, having one layer of endosperm e.g. Arabidopsis (a) or the endosperm might extend to several layers e.g. tobacco (c). Note the spatial distribution of the embryo (shades of green) and endosperm (grey and black) in the four seeds.

Table 2.6: Examples of promoters used for seed specific expression of recombinant proteins (other than antibodies)

Plant specie	Promoter	Recombinant protein	Subcellular localization / plant tissue	Reported expression level
Wheat	Ubiquitin-1	Human serum albumin	Endoplasmic reticulum (ER) derived protein bodies	0.5% TSP
Maize	27-kDA γ-zein	*E. coli* heat labile toxin (LT-B)	Starch granules	
Rice	Gt13a, endosperm specific	Human serum albumin	Endosperm	2.75 g/ kg of brown rice
	Glutein GluB-1	7Crp	ER derived protein bodies (PB)	60 µg/grain
	Glutein GluB-1	Human interleukin	ER	4.5 mg/100g
	Glutein GluB-1	Cholera toxin B subunit	PB	30 µg per seed
Soybean	Soybean glycinin promoter	Heat labile toxin (LT-B) of enterotoxigenic *E. coli*	ER derived PB	2.4% TSP
	Glycinic G1	Human basic fibroblast growth factor	Seed	2.3%
	α – subunit of the β –conglycinin promoter	Human coagulation factor IX	Protein storage vacuole (PSV)	0.8 g/kg seeds
Safflower	Phaseolin promoter	Apolipoprotein al milano	Apoplast	7.3 g/kg seeds
Tobacco	Soybean 7S globulin promoter	Human acid β-glucosidase	PSV	3% TSP
Arabidopsis	Phaseolin promoter	Human α-L-iduronidase	ER	18 µg/mg TSP

Table has been adapted from Khan *et al.*, 2012

few layers of endosperm like in case of tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*) (Figure 2.3, c) or reduced to a single cell layer like in mouse-ear cress (*Arabidopsis thaliana)* (Figure 2.3, a) (for a quick reference guide on seed anatomy see www.seedbiology.de).

The endosperm and the cotyledons of the embryo are the storage compartments and store energy rich nutrients for germination. Depending on the plant specie, the nature of these stored energy sources vary, i.e. in case of oil seeds, lipids as triacylglycerol are stored in subcellular oil bodies; the endosperm of cereals (monocots) predominantly bear carbohydrates (starch) followed by proteins, while soybean and pea have protein rich cotyledons. For molecular farming of valued proteins, these storage compartments are predominantly triggered using specific promoters. Tables 2.6 and 2.7 summarised various such promoters used for in seed expression of heterologous proteins.

The expression of antibody or antibody-fragments in the seed can be achieved by the constitutive viral promoters e.g. 35S promoter of the cauliflower mosaic virus (35S CaMV). However although this promoter can yield high amount of antibodies in various plant tissue, its strength in driving expression in seeds is poor, and a seed-specific promoter can lead to high accumulation (De Jaeger *et al.*, 2002; De Wilde, 2012; Petruccelli *et al.*, 2006). A murine full length antibody called 14D9 when expressed under the control of the seed-specific promoter β-conglycinin (derived from soybean) lead to accumulation of about 1 % of TSP in tobacco seeds, while the same heavy and light chains driven by the 35S CaMV promoter expressed functional antibody to about 0.4 % of TSP in seeds and up to 5% of TSP in leaves of tobacco.

It is reasoned that the seed-specific promoters are usually active during the seed filling stage (development and maturity) while the 35S CaMV ceases to express after the initial stages, thus accounting for lower accumulation (Chen *et al.*, 1986; Petruccelli *et al.*, 2006). Similar trend was also observed for other studies expressing ScFv-Fc for application other than therapeutic use. A ScFv-Fc against maltose binding protein (MBP) when expressed in Arabidopsis under the control 35S CaMV promoter or the seed-specific β-phaseolin promoter yielded only high amount of functional anti-MBP ScFv-FV with seed-specific β-phaseolin promoter

(De Wilde, 2012). While a ScFv against dihydroflavonol-4-reductase of petunia, which expressed to 1% of TSP under the control of 35S CaMV expressed up to 36% under the control of β phaseolin promoter in Arabidopsis seeds (De Jaeger *et al.*, 2002). Besides the β-phaseolin and β-conglycinin promoters mentioned so far, several different seed-specific promoters like- USP (unknown seed promoter)(Zimmermann *et al.*, 2009), glutelin-1 (gt-1) promoter (Ramessar *et al.*, 2008), legumin A-promoter (Perrin *et al.*, 2000), maize ubiquitin promoter, etc. have been evaluated for expression of antibodies and other therapeutic proteins in plants (Khan *et al.*, 2012) (Tables 2.6 and 2.7).

Table 2.7: Few examples of in seed produced antibodies for therapeutic use

Plants	Antibody type and pathogen against	For treatment of	Seed compartment	Accumulation level	Promoter	Reference
Maize	Full length mAb Anti-HIV (2G12)	Human	ER / embryo	38-75 µg/g	ER specific rice promoter	(Rademacher *et al.*, 2008)
Maize	Full length mAb Anti-HIV (2G12)	Human	ER and protein body	5.7 % TSP	Glutelin-1 (gt-1) promoter	(Ramessar *et al.*, 2008)
Wheat and rice	ScFv (anti-carcinoembrionic antigen)	Human	Endosperm	30 µg/g	Ubiquitin-1 promoter	(Stoger *et al.*, 2000)
Pea	ScFv Anti-F4+ETEC (BA11)	Piglets	Seeds	2 g/kg	USP	(Saalbach *et al.*, 2007)
Pea	ScFv (anti-Eimeria)	Chickens	Seeds	1.76 mg/g	USP	(Zimmermann *et al.*, 2009)
Rice	ScFv(Anti-K99+ETEC)	Cattle	Embryo	(Low expression)	CaMV	(Sunilkumar *et al.*, 2009)

Abbreviations: ER-Endoplasmic reticulum, CaMV 35S- Cauliflower mosaic virus 35S promoter, USP- unidentified seed protein from *Vicia faba.* The mAb- monoclonal antibody, ScFv- single chain variable fragment, K99+ETEC- enterotoxigenic *Escherichia coli* bearing K99 fimbriae, F4+ETEC- enterotoxigenic *E. coli* bearing F4 fimbriae. NB: Purification of antibodies is mostly foreseen for the antibodies intended for human application while for animal diseases its predominantly aimed for oral administration as semi-processed crude seed flour.

Overall it has been seen that promoters specific for endosperm in case of the monocot seeds and those specific for cotyledons (i.e. the embryo) in case of dicot seeds generally lead to high expression (Khan *et al.*, 2012). Both these respective compartments are the protein sinks of the seed. Compartment specific promoter though play a big part in high accumulation, they are not the sole factor

(Drakakaki *et al.*, 2006; Streatfield, 2007), since subcellular trafficking and accumulation play an equally important role. The endomembrane system of seeds differs from the other vegetative tissues. The seed cells have the specialised subcellular structures called protein bodies (PB), protein storage vacuoles (PSV), starch granules and oil bodies, which make up the vaults of storage sink. Some of these organelles like PSV and PB allow stable storage of antibodies, influencing its stability, accumulation and thus overall yield (Khan *et al.*, 2012; Stoger *et al.*, 2000). However targeting of the recombinant proteins to a particular subcellular compartment can be tedious. The trafficking of native storage proteins in itself is highly convoluted, and differs for individual storage protein and the plant species (Khan *et al.*, 2012) (see schematic representation, Figure 2.4). For instance, globulins and albumins are predominant storage proteins of many seeds, which usually reach the end destination of PSV from the endoplasmic reticulum (ER) *en route* the Golgi apparatus via dense vesicles, however in pumpkin seeds these proteins reach PSV by omitting the Golgi apparatus but instead traffic through precursor-accumulating (PAC) vesicles (Hara-Nishimura *et al.*, 1998; Hohl *et al.*, 1996) (Figure 2.4). Our group previously demonstrated by expressing different single chain-Fc antibody chains in Arabidopsis seeds, that high expressing antibodies were aberrantly localised. A part of the accumulated antibody was retained in the ER-derived vesicles while antibodies were detected in the periplasmic space (PS) between the plasma membrane and the cell wall. This also disrupted the localisation of endogenous ER chaperons like BiP and calreticulin, which were atypically also found in the PS (Van Droogenbroeck et al., 2007). Despite these complications in targeting of recombinant proteins in seeds, using some general strategies like signal peptide of endogenous seed proteins, retention signals like KDEL etc. several recombinant proteins have been successfully expressed and stably accumulated in seed storage organelles.

Addition of a KDEL tag, for retention within the ER also leads to higher accumulation in seeds (De Jaeger *et al.*, 2002; Peters and Stoger, 2011). Secreting antibodies (by addition of N-terminal signal peptide), and retaining them within the endo-membrane system (C-terminal KDEL/ SEKDEL peptide) in general is a preferred strategy for in seed antibody production. The specific retention also

enables for control over the post translational modification like addition of glycans (Loos *et al.*, 2011a). The glycosylation of proteins until the ER, in plants and animals (also yeast and basidiomycetes) is similar (Berends *et al.*, 2009). Thus by retention in ER one can refrain for addition of plant specific glycans, especially in cases where glycosylation is important for efficacy like ADCC in case of passive immunisation therapy or intravenous administration.

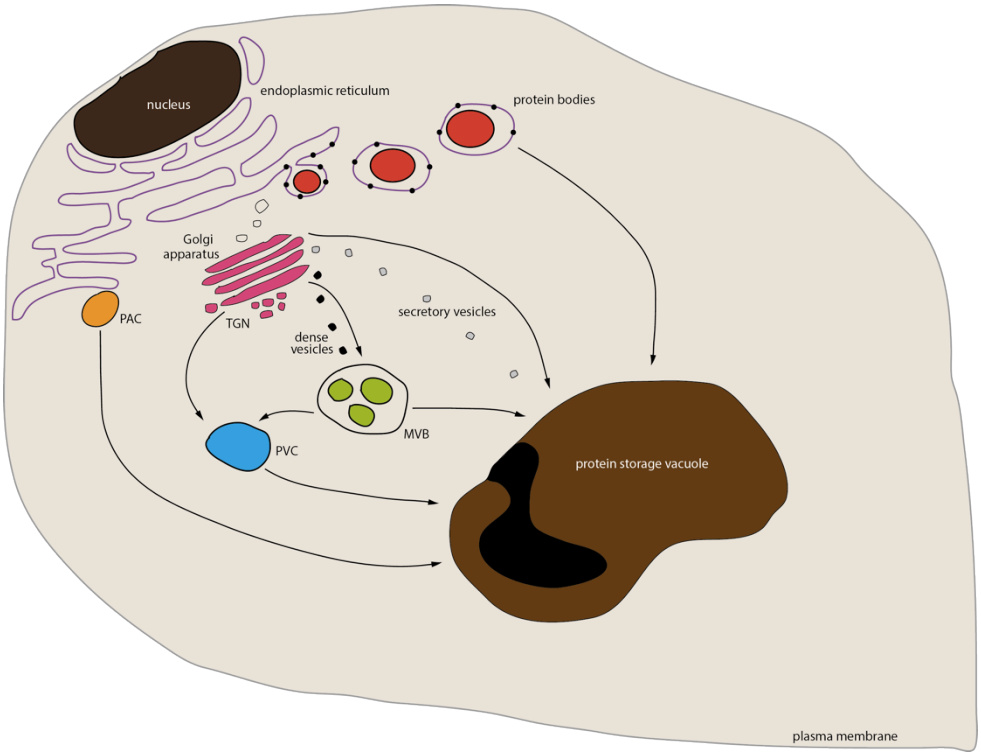


Figure 2.4: Schematic representation of protein trafficking in seeds to the storage compartments. The predominant destination of the endogenous storage proteins and most recombinant proteins is the protein storage vacuoles (brown) or the endoplasmic reticulum derived protein bodies (red). The proteins may traverse in vesicles through various alternative paths in the endospermic and embryonic tissue, either through the Golgi apparatus, or forming multi-vesicular bodies (MVB) (green), or pre-vascular compartment PVC (,blue), or through precursor-accumulating vesicles PAC (yellow). For some examples and more details see the text. The figure has been adapted from Khan *et al.*, 2012.

Further it has been suggested through oral feeding experiments that the bio-encapsulation in endomembrane protects from the harsh gastric environment on oral delivery of crude or semi processed seed meal (Takagi *et al.*, 2010; Zimmermann *et al.*, 2009). Protection at the mucosal surface can be achieved

effectively by topical in feed/food administration of antibodies (Streatfield, 2006). The protective role of oral feed based passive immunisation has been proven for coccidiosis in chicken and post-weaning diarrhoea in piglet, both these animal disease bearing high economic burden (Saalbach *et al.*, 2007; Zimmermann *et al.*, 2009). A protozoan of Eimeria genus causes the coccidiosis in chicken. When pea seeds bearing anti-*Eimeria* ScFv were forced fed to chicken it protected them from subsequent *Eimeria* challenge. It was noticed that the protection was only conferred by in feed administered antibody, while purified antibodies (produced from tobacco leaves) failed to achieve similar protection. This discrepancy was due to the protection offered by the pea seed matrix from gastric proteases which resulted in 100-fold gain in ScFv stability (Zimmermann *et al.*, 2009). Similarly protective effect or in feed based prophylaxis was also reported for prevention of ETEC caused post-weaning diarrhoea in piglets. The piglets were not only protected, but a detailed autopsy revealed that the antibodies were retained at the gastric mucosal surface and were not detected in other organ like heart, lungs etc. (Saalbach *et al.*, 2007). Crude crushed seed based passive oral immunisation is an attractive strategy for farm animals, and though not explored yet for humans would be interesting application in future. A few examples of antibodies for human application (therapeutic and diagnostic) are listed in Table 2.7. Seed made antibodies are yet to reach the clinical phase, given the current regulatory framework this might take some time, and perhaps in comparison seed made antibodies for animal use might be available before the ones for human application.

Conclusion

Despite the several merits of plants as antibody expression systems there are only a few antibodies that have made it through clinical trial phase of which tobacco produced CaroRX™ the chimeric secretory IgA antibody has been approved for clinical use in E.U. (Obembe *et al.*, 2011). One of the chief reasons for delay in bringing plant made therapeutic antibodies to the market can be due to regulatory issues associated with genetically modified plants, and the already established mammalian systems for monoclonal mAb systems (Paul *et al.*, 2011). Due to which most big pharmaceutical companies have been hesitant in

investing. Contamination of the environment with GM plants can be a serious issue. In the past there has been an unfortunate event involving Prodigene (Texas, US), a plant biotechnology company, which had a promising vaccine produced in maize against transmissible gastroenteritis virus of piglet, which had reached to phase I status (Basaran and Rodríguez-Cerezo, 2008). However, following contamination of non-GM corn crop the company was charged of violating the plant protection act (Fox, 2003)(see the article 'The Prodegene incident'[7]), consequentially the company winded up its activities. This had an undesired negative effect on the community at large. However things are now changing mostly due to the development of latest expression technologies that have enabled transformation of several different plants some of which can grow fast and produce large amount of biomass in short time. Along with this, the expression vectors have been improved which enable high levels of antibodies production from reasonably small plant biomass (Paul and Ma, 2011; Whaley *et al.*, 2011; Xu *et al.*, 2011a). This means that substantial amount of antibody can be harvested from a limited number of plants that can be grown in contained glass facilities rather than on large open fields as initially anticipated.

There has been a paradigm shift in the industry and the regulatory bodies as well. Now there are a number of companies producing vaccines and antibodies in plants, bringing products close to clinical phase. The European Medicine Evaluation Agency has published a European guideline encompassing several issues about recombinant protein production in plants (CHMP/BWP/48316/2006). This brings hope that in the near future the full potential of plants as a cost effective platform would be realised.

[7] http://www.fas.org/biosecurity/education/dualuse-agriculture/2.-agricultural-biotechnology/prodigene-incident.html

Chapter 3

Enterotoxigenic *Escherichia coli* caused post weaning diarrhoea

The pathogen, pathogenesis and prevention

Vikram Virdi, Henri De Greve and Ann Depicker

V.V. wrote the chapter, H.D.G and A.D edited it.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) caused post-weaning diarrhoea (PWD) is a common piglet disease, which mainly occurs in the first week of weaning, but is common until the 3rd weak post weaning (Amezcua *et al.*, 2002; Fairbrother *et al.*, 2005). Suckling piglets are protected from the ETEC infection by the anti-ETEC specific antibodies in the sow's milk. The placenta in pig does not permit the transfer of immunoglobulin from pregnant sow to the developing pig fetus. Thus the maternal colostrum and milk are the only source of protective antibodies. At weaning this lactogenic immunity is lost and in addition the weaning period induces several stresses on the young piglet. These basically include psychological stress of separation and physiological stress due to change of diet; both these conditions consequentially negatively influence the immune status of the piglet. It has been reasoned that the ETEC infection in conglomeration to these stresses leads to PWD. Severely affected piglets often die preceding short illness (Vanbeersschreurs *et al.*, 1992), most newly-weaned piglets however suffer from a benign form of the disease (symptoms in Table 3.1). Piglets, who recover from this morbidity, have lower feed consumption and weight gain (Fairbrother *et al.*, 2005). Both, the death and lower weight gain results in heavy economic loss (Amezcua *et al.*, 2002). Porcine derived product have far reaching applications beyond its primary consumption as human food (Christien, 2010); thus a set back to the pork production has global repercussions. In the western hemisphere where porcine farming is practised at an industrial to semi-industrial scale, PWD can lead to losses of millions of dollar per annum. In Belgium it has been estimated that a mortality rate of 2% leads to an annual loss of EUR 15 million, which significant affects the profit margin and earnings of porcine farmers. While on the other side of the globe, in the developing world— post-weaning diarrhoea can have detrimental effect on the basic livelihood of a subsistence farmer. It has been envisioned that encouraging rural household porcine farming will help in eradicating rural poverty in countries with developing economies e.g. Vietnam in Southeast Asian, and Kenya in Sub-Saharan Africa (Costales *et al.*, 2005; Githigia *et al.*, 2012). Post-weaning diarrhoea is a hurdle in reaping these economic benefits. The economic losses

cannot be avoided at present since there is no vaccine or a safe therapeutic against this disease. The global disease burden of PWD has seemingly worsened in the recent years; reason for this could be the ban on the prophylactic use of antibiotics in the EU (which was previously a standard practice) or due to recent change in animal husbandry practices, or perhaps evolution of more pathogenic strains of ETEC, etc.

Given the grievousness of this disease in porcine industry several strategies are being developed. These strategies either focus on the ETEC pathogen i.e. developing vaccines and passive immunisation therapies, use of plant or yeast derived products that impede the bacterial infection, or the feed management- which basically aims to maintain the gastric physiology and barrier function, while other solutions aim at better animal management. In this following mini-review, we unfurl the cause and treatment issues regarding ETEC causing PWD in piglets, with emphasis on predominantly isolated F4 fimbriae bearing ETEC.

Table 3.1: Symptoms of ETEC caused PWD in newly weaned piglets

- Bluish-red discolouration of ears and abdomen in acute cases
- Generally, drop in feed consumption
- Greyish-yellow watery diarrhoea
- Rectal temperature usually normal
- Dehydration and depressed
- Post mortem- cadavers generally cyanotic and dehydrated

  Small intestine dilated, oedematous and hyperaemic

The ETEC Pathogen

The two important hallmarks of PWD causing ETEC strains are that they produce adhesins and enterotoxins. The adhesins are important for attachment to specific intestinal receptors; this attachment facilitates the colonisation of the bacteria, followed by secretion of the enterotoxin in the intestine that consequentially leads to diarrhoea (Fairbrother *et al.*, 2005; van Beers-Schreurs *et al.*, 1992) (Figure 3.2). The most common isolated ETEC strains from piglets with PWD either bear the F4 and at times the F18 fimbriae, these serve as both virulence

markers and virulence factors (Fairbrother *et al.*, 2005). The fimbriae are long flexible proteinaceous appendages that extensively cover the ETEC surface. The fimbriae are expressed at 37°C and not at 18°C, i.e. on infection in piglet gut and not in the environment (Fairbrother *et al.*, 2005).

The F18 fimbriae have a zigzag pattern and are about 1 to 2 µm long (Hahn *et al.*, 2000). There are 5 genes that encode for the F18 fimbria and are present on a plasmid, these genes are– *fedA,* (which codes for the major pilus subunit), *fedB* (outer membrane protein usher), *fedC* (chaperone), *fedE* (minor protein) and *fedF* (adhesin, present on the tip of the fimbria) (Imberechts *et al*., 1996). The F18 fimbriae occur in two antigenic variants F18ab and F18ac, which can be distinguished in epidemic samples by serology (Nagy and Fekete, 1999). The tip adhesion FedF encoded by the *fedF* gene is highly conserved and hence the FedF adhesin is suggested as suitable target for vaccine development (Smeds *et al.*, 2003) or an anti-FedF antibody as a passive immunisation therapy might also be worth evaluating for impeding F18 bearing ETEC ($F18^+$ETEC) infection. Both of these variant of $F18^+$ETEC strains, most predominantly bear heat-stable toxins STa and STb and only rarely the heat-labile toxin (LT) were isolated; while in case of F4 bearing ETEC strains LT is the predominant toxin (Fairbrother *et al.*, 2005; Rippinger *et al.*, 1995). The receptors for the attachment to these F18 variants are present on the intestinal epithelium of piglets older than 3 weeks of age. Hence $F18^+$ETEC are associated with PWD.

The F4 bearing ETEC infections unlike F18 are seen in neonatal, nursing piglets and post weaning. The F4 fimbriae occur in 3 variants classified as F4ab, F4ac and F4ad according to the major subunit and adhesion– FaeG. The FaeG has a constant antigenic subunit ‘a’ and variable domains ‘b’, ‘c’ or ‘d’ (van Zijderveld *et al.*, 1990). The three variants of F4 are not just serotypically distinct but they also show differential specificity to intestinal receptors. Some piglets are susceptible to all the three variants, while others can be susceptible to either two variants (either F4ab and F4ac or F4ab and F4ad) or a single variant (Francis *et al.*, 1999). Though all the three variants have been reported in different epidemiological survey, over all the F4ac variant is globally most predominant (Fairbrother *et al.*, 2005; Osek, 1999).

A large, non-conjugative plasmid bears the operon of 10 genes that encode the F4 fimbriae, which are named *FaeA* to *FaeJ*, of which the gene *faeG* is coding for the major adhesion protein FaeG. The other fimbrial proteins encoded by this F4 fimbrial gene cluster include— FaeD, which is an outer membrane usher and also serves as the anchor molecule; FaeC, FaeH, FaeF are the other structural units, and are regarded as the minor units while FaeE is the chaperon protein. The research in the field of these F4 fimbriae in the last 3 decades has helped in unravelling their structure and biosynthesis, which has been summarised by Verdonck *et al.* (2004), a brief description of which is as follows.

The biogenesis of the F4 fimbriae is influenced by several environmental factors like temperature, pH, carbon source, oxygen level, osmolarity, etc. The optimum conditions for F4 fimbriae expression are at 37°C, towards the end of exponential growth phase and when within the pH range of 6.5 to 8 (Verdonck *et al.*, 2004a).

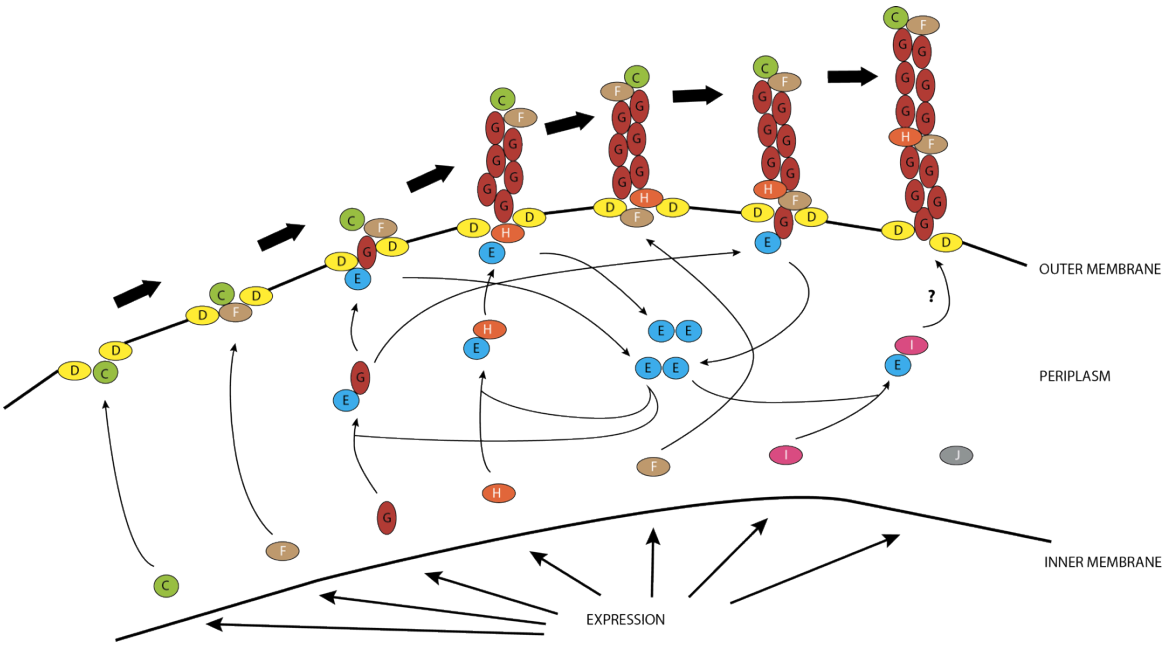


Figure 3.1: Biogenesis of F4 fimbriae. Each of the fimbrial subunits (FaeC- green, FaeF-brown, FaeG-red, FaeE-blue, FaeH-orange, FaeI-pink) has been represented as an oval. The fimbrial assembly starts at the outer membrane FaeD usher with the translocation of FaeC, which form the tip followed by FaeF. The major subunit and adhesin FaeG forms the predominant part of the filamentous F4 fimbriae. Previous models suggest that the multiple FaeG adhesion are interspersed with limited units of FaeH and FaeF, however recent structural data argues the incorporation of FaeF and FaeH among the multiple FaeG units. The free FaeE occurs as a dimer in the periplasm and as monomer it acts as the chaperon which transports the Fae subunits across the periplasm as indicated with arrows. The involvement of FaeI in the fimbrial biogenesis is suggested but its exact involvement is uncertain yet (indicated by '?' mark). Figure adapted from Van den Broeck *et al.*, 2000; Van Molle *et al.*, 2009.

Under apt conditions, the fimbrial assembly starts with the translocation of the fimbrial subunits across the inner membrane. The conserved characteristic signal peptides on the N-termini of these Fimbrial units (Fae) enable their transit through the secretory pathway into the periplasmic space. The FaeD unit is 82,126 Da protein that anchors into the outer membrane and acts as the assembly platform. Next the usher FaeD translocates the FaeC subunit (16,900 Da), which is destined to form the tip of the fimbria, after which FaeF (15,200 Da) follows. The FaeF provides as an adapter molecule for attachment of the major subunit (also adhesin) FaeG to the FaeC at the tip. Predominantly the major subunit FaeG and few molecules of minor subunits FaeF and FaeH make up the prominent portion of the F4 fimbrial shaft (Figure 3.1). The FaeE (24,768 Da), which occurs as a dimer in solution, chaperons the FaeG, FaeH, and FaeF units (Mol and Oudega, 1996; Mol *et al.*, 1996). One after the other several units of FaeG are brought to the FaeD by the FaeE (E-G assembly); FaeD then translocates the FaeG molecule, progressively protruding the (FaeC-FaeF-FaeG-FaeG…) tip assembly (Mol *et al.*, 2001). The Fae subunit-subunit connections are achieved by donor strand exchange mechanism and are very specific, the protruding N-terminal from an incoming Fae extends in place of the 'G' β-strand of the preceding Fae molecule (Barnhart *et al.*, 2003). The N-terminal donor peptide is very specific, and determines the specific FaeC-FaeF, FaeF-FaeG or FaeG-FaeG connection. Since the interaction of FaeG-FaeG units is very specific it is not know how stoichiometrically FaeF and FaeH units would be interspersed among the multiple FaeG units, as it has been suggested in the traditional F4 fimbriae model (it is probable that some units of FaeI are also incorporated (Bakker *et al.*, 1992b)). The process is schematically represented in Figure 3.1

The combination of the virulence factors inclusive of the fimbriae and the toxins define the serotypes of ETEC associated with PWD. Diarrhoea causing ETEC strains may have other virulence factors as well, like the presence of alpha-hemolysin. This cytotoxin can damage a wide range of cells and its presence is detected in most recent isolated PWD samples (Heo *et al.*, 2012; Smith and Linggood, 1971; Wu *et al.*, 2007). The serotype O149 bearing the F4 fimbriae is the most frequently isolated, other predominant serotypes are listed in Table 3.2 (Heo *et al.*, 2012). The prevalence of different serotypes differs geographically,

and also the pathogenicity of the same serotype may differ. For instance the serogroup O139 is isolated in different epidemiological studies globally; in Australia the serogroup is associated with PWD while in Europe it is reported to cause oedema disease (Fairbrother *et al.*, 2005).

Table 3.2: Some commonly isolated ETEC serotypes from piglets with PWD

O serotype	Associated fimbrial antigen
8	F4ab, F4ac
138	F18, F4ac
139	F18
141	F18, F4ab, F4ac
147	F4ac, F18
149	F4ac, F18
157	F4ac

Table reused from Heo *et al.*, 2012

Pathogenesis and factors affecting ETEC related PWD in piglets

The ETEC related PWD disease is a multifactorial disease, where ETEC infection among the several predisposed weaning induced stresses (described later) causes acute post weaning diarrhoea. There are three important steps for the ETEC to cause PWD- they are– attachment, followed by proliferation and secretion of toxins (Heo *et al.*, 2012; van Beers-Schreurs *et al.*, 1992) (Figure 3.2).

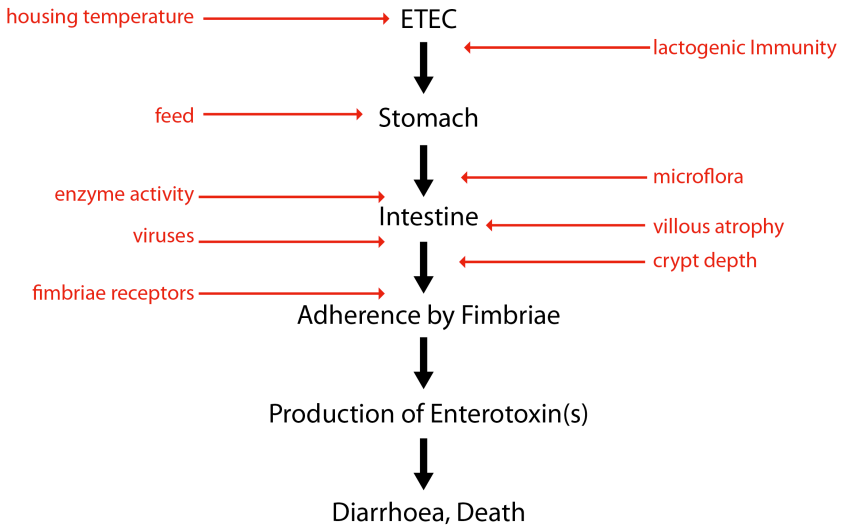


Figure 3.2: Overview of the steps in the pathogenesis of ETEC caused PWD. The various cofactors that influence the establishment of ETEC are indicated (in red). More details are in text. The figure is adapted from Fairbrother *et al.*, 2005; van Beers-Schreurs *et al.*, 1992.

The ETEC pathogen seemingly spread through feed, or by other piglets, or animals, contaminated faeces or even through aerosols. The ETEC bacteria are usually present in the immediate environment of the pig farm; getting rid of the ETEC is very difficult to impossible. ETEC in dung can survive for up to 6 months, fumigation techniques does not affect the bacteria beyond the depth of a quarter of an inch (0.6 cm) (van Beers-Schreurs *et al.*, 1992). Also the ETEC can be transmitted through aerosols; it has been seen that temperature influences the half life of the bacteria and the concentration, which is higher at 15°C than at 30°C (van Beers-Schreurs *et al.*, 1992). The concentration of the ETEC is very important in disease presentation. In experimental model 10^9 to 10^{10} ETEC bacteria are needed to induce diarrhoea (Cox *et al.*, 1991). In another study artificial contamination of the floor with 10^6 colony-forming units (cfu/cm^2) did not lead to diarrhoea (Melin *et al.*, 2000). The environment of the piglets in natural setting does not have a high titer of ETEC. It is on infection and attachment that the ETEC bacteria proliferate in the intestine (van Beers-Schreurs *et al.*, 1992). Once the ETEC colonise the intestine they secrete one or more toxins. The LT toxin consists of an A subunit and five B subunits; the B subunits bind to the intestinal GM1 ganglioside and enables a pore formation through which the A subunit penetrates the host cell (Field *et al.*, 1989). The LT toxin leads to activation and increased production of the cyclic adenosine monophosphate (an intercellular messenger molecule), and further leading to increase in the secretion of sodium chloride, bicarbonates and water into the intestinal lumen (Heo *et al.*, 2012; van Beers-Schreurs *et al.*, 1992). The ST toxin on the other hand leads to absorption of liquids and salts by activating the production of cyclic guanosine monophosphate. The net effect of both these toxins is the hyper secretion of salts and water in the intestine. Under such situations, the distal part of the alimentary canal is incapable of reabsorbing these high amounts of liquids and salts which results in diarrhoea (Figure 3.2), along with other symptoms as mentioned in Table 3.1.

Though ETEC is the key element in PWD, its colonisation leading to disease is only possible under the presence of certain predisposed elements (marked in red in Figure 3.2). Most important is the **presence of serotype specific receptors**. As mentioned above, the $F4^+$ETEC serotypes, each has a unique

receptor in the small intestine (Van den Broeck *et al.*, 2000), likewise the F18^{+}ETEC bacteria also have its specific receptors. In nature piglets devoid of one or more of these receptors exist; such piglets are naturally insensitive to F4^{+}ETEC infections. The phenotypic difference has been demonstrated to be genetic in nature, which is inherited in accordance with Mendelian laws, as a dominant allele (Sellwood, 1979)**.** Hence, in principle determination of receptor negative or positive pigs should be possible for all variants of ETEC. Jørgensen *et. al.* developed a DNA marker test based on the Mucin4 gene polymorphism for genotyping of the F4ac/ac resistant or susceptible piglets (Patent no. WO2004/048606-A2)**.** However a later study by another group compared the sensitivity of this test with *in vitro* villous adhesion test (which is a gold standard) and showed that the result were 100% affirmative for F4ab but correlated only for 24% in case of F4ac (Rasschaert *et al.*, 2007). In addition involvement of more than one receptor for variant specific F4^{+}ETEC sensitivity is suggested. Overall determination of resistant or susceptible piglets is not that straightforward. Along with adhesive and non-adhesive phenotype, week adhesive phenotypes also exist (Van den Broeck *et al.*, 2000). Probable reason for this variation in phenotypic expression of receptors is said to be due to gene epistasis, or inhibition of or alteration of receptor expression (Bijlsma and Bouw, 1987). The expression of F4 receptors also varies with age. In an earlier report it was shown that susceptible newborn piglets expressed only 1/16th of the amount of receptors expressed in 35 day-old unweaned piglets (Conway *et al.*, 1990). However Willemsen and de Graaf's report published in 1992 contradicted the previous observation, stating no difference was noted between the piglets of 7 day and 35 days of age (Willemsen and de Graaf, 1992). Van den Broeck *et al.* summarised several such studies comparing the susceptibility and the extent of receptors, over all it seems that the highest number of receptors are usually detected in piglets 1-5 weeks of age (Van den Broeck *et al.*, 2000).

In addition to the variable expression of the receptors, and perhaps involvement of more than one putative receptor for F4^{+}ETEC infection, 'the receptor(s)' as per traditional physiological definition of a receptor (capable of inducing signal on binding) for F4^{+}ETEC is not yet fully characterised. There are more than 30 publications investigating the putative receptors; some of the general consensus

remain that the F4 fimbriae interact with a glycolipid or the glycolipid is a part of the receptor present on the mucosal surfaces, especially the small intestine. The receptor characterisation work until early 2000 has been reviewed by Van den Broeck *et al.* (2000), which basically concluded that a 'β-galactose is involved in the binding of the F4 fimbriae' and that the glycan is either linked to a surface glycolipid or a glycoprotein. Further, the glycoprotein that interact with F4ab have a molecular weight of 25, 30, 40-42 and 60 kDa in intestine while 16, 40-70, 74, 210 and 240 kDa in brush border membranes. The glycoproteins that recognise F4ac have a molecular weight of 25, 30 and 60 kDa in intestinal mucosa; 40-70, 210 and 240 kDa in brush border membranes. While the third variant F4ad recognised 45-70 kDa glycoproteins in brush border membrane (Van den Broeck *et al.*, 2000). Some of the recent progress made in deducing the exact interacting glycoconjugates, employs state of the art mass spectrometry techniques in complement with thin-layer chromatography and affinity chromatography. To identify the minimal recognition segment for F4ac, F4ab and F4ad fimbriae, Granger *et al*. used porcine serum transferrin (pSTf) and purified glycosphingolipids (GSL)(Grange *et al.*, 2002). They discovered that only the F4ab binds with high affinity to pSTF, in which it binds to a single N-glycan and more precisely to a core *N*-acetylglucosamine. While results with GSL interaction study showed that all three variants bind to a β-linked *N*-acetylhexosamine, and that the presence of a β-linked terminal galactose (*N*-acetylglucosamine or *N*-acetylgalactosamine) resulted in stronger adhesion (Grange *et al.*, 2002). In another study by Coddens *et al.;* the authors discovered distinct carbohydrate binding pattern to the three variants of free F4 fimbriae as well $F4^{+}$ETEC, using glycosphingolipids from erythrocytes and from porcine small intestinal epithelium. The F4ac (fimbriae as well as bacteria) bound galactosylcermide (Galβ1Cer) with spingosine and hydroxyl 24:0 fatty acid, while F4ab (Fimbriae and bacteria) bound to galactosylceramide sulfatide (SO3-3Galβ1Cer), sulf-lactosylceramide (SO3-3Galβ4Glcβ1Cer), and globotriaosylceramide (Gala4Galβ4Glcβ1Cer) with phytosphingosine and hydroxy 24:0 fatty acid. While the F4ad bound to gangliotriaosylceramide (GalNAcβ4Galβ4Glcβ1Cer), gangliotetraosylceramide (Galβ3GalNAc4Galβ4Glcβ1Cer), isoglobotriaosylceramide (Gala3Galβ4Glcβ1Cer),

and neolactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer) (Coddens *et al.*, 2011). Recently, by comparative proteomic analysis of brush border proteins from resistant and sensitive piglets one of the F4 receptors has been identified to be an aminopeptidase N (pAPN) (Melkebeek *et al.*, 2012). The sialic acid bearing carbohydrates on the pAPN seemingly determine the attachment and its subsequent clathrin-mediated endocytosis (Melkebeek *et al.*, 2012). Future determination of all the other F4 receptors would help in developing anti-ETEC therapeutics or even developing of convenient non-invasive or minimal invasive test methods for selection of resistant piglets for breeding programs; but the issue of variation of phenotypic expression of these different receptors might prove it to be a difficult task.

Then there are **other factors** that are equally vital for establishing ETEC infection. According to the current pig rearing practices the piglets are weaned at 3-4 weeks of age (body weight 4-5 kg) as compared to the traditional weaning age of up to 5 weeks (body weight 5-10 kg)(different countries have their stipulated minimum suckling period)[8]. The incentive for **early weaning** is that it enables higher piglet production per sow per annum. This separation of piglets from sow and deprival of milk is a dramatic transition for the young piglets leading to psychological, physiological and immunological stress. The gut of piglets, weaned at this age, show villous atrophy (i.e. erosion of the absorptive villous surface) and crypt hypertrophy as a result of the changes in diet and stress (Kenworthy, 1976). Reduction in villous height, and increase in crypt depths, leads to compromising of the barrier function and the general mucosal immune status of the intestine. In addition to this, the unavailability of protective factors from the milk including ETEC specific antibodies, iron binding lactoferrin and transferrin that impede bacterial growth, provides opportunity for ETEC to colonise the small intestine. Rotavirus co-infection with ETEC is another important cofactor in PWD (Lecce, 1983; Pluske *et al.*, 1997). In fact in a certain experimental piglet disease model to study ETEC and PWD, the piglets are deliberately inoculated with rotavirus (Harmsen *et al.*, 2005).

[8]http://www.thepigsite.com/

In addition to these, other major cofactors enabling the ETEC infection are the **dietary composition and the housing conditions**. Piglets when weaned at 3 weeks of age do not have the necessary enzymes to digest the solid feed resulting in proliferation of saccharolytic or saccharo-proteolytic flora, which leads to increase in haemolytic *E. coli* in the gut. Also hypersensitivity to feed components has been reported, which lead to villous atrophy and intestinal alterations. The temperature in piglet housing units also influences the ETEC growth, stability and thus infection pressure. After the ban on prophylactic antibiotic use, protective therapy in case of ETEC related PWD is predominantly focused on either modulating these cofactors or developing specific anti-ETEC molecules, as described in subsequent section.

Prevention strategies for ETEC related PWD

The predominant prophylaxis until a decade ago was the use of growth promoting antibiotics. The regular use of sub-therapeutic dose of these antibiotics was suggested to control ETEC infection, also addition of these in feed was seen to have a positive effect on the overall feed intake and weight gain. However now the prophylactic use of such growth promoting antibiotics is banned in the EU and discouraged in many countries globally. This prohibition has had two detrimental consequences, the increase in PWD incidences and the increase in therapeutic use of antibiotics (Heo *et al.*, 2012). The risk of introducing antibiotic resistance has led to the need for development of a suitable safe alternative (Adjiri-Awere and Van Lunen, 2005; Vondruskova *et al.*, 2010). To this avail, several anti-ETEC specific approaches like vaccines and passive immunisation therapies, as well as other non-antimicrobial compounds have been tested. However, there has been no 'magic bullet' to combat ETEC related PWD yet (Fairbrother *et al.*, 2005; Heo *et al.*, 2012).

Some of the major products experimented with for preventing PWD include the use of metallic compounds like zinc oxides, organic acids, different composition of dietary proteins, probiotics, prebiotics, anti-ETEC antibodies and subunit vaccines (Fairbrother *et al.*, 2005; Heo *et al.*, 2012; Katouli *et al.*, 1999; Melin and Wallgren, 2002; O'Doherty *et al.*, 2005; Roselli *et al.*, 2005; Vondruskova *et al.*, 2010).

Zinc is an essential micronutrient in the piglet diet, the deficiency of which is suggested to have retarded growth and reduction of several enzyme (e.g. zinc metaloenzymes, alkaline phosphatase) (Katouli *et al.*, 1999; King, 2011; Heo *et al.*, 2012)(Katouli *et al.*, 1999)(Katouli *et al.*, 1999) (Katouli *et al.*, 1999) (Katouli *et al.*, 1999). Though the dietary recommended level of zinc is 100 mg/kg body weight of weaned piglets, the use of zinc up to 30 times higher has shown to have an effect on the prevention of PWD (Heo *et al.*, 2012; Højberg *et al.*, 2005). It is suggested that the action of zinc in preventing PWD might be due to it direct antimicrobial action e.g through interaction with sulphur atom in bacterial proteins, along with provoking the host system, like stimulation of anti-microbial peptide secretion in the small intestine, or influence on the commensal gut microbiome that is advantageous in prevention ETEC colonisation, or Zn might inducing growth factors, etc. The actual mechanism of zinc in relation to PWD so far remains speculative (Heo *et al.*, 2012). There is also a concern about the excessive use of zinc, in regards to the toxicity and the effect it would bear on the environment, hence many countries have reservations on the excessive use of zinc in the feed of newly weaned piglets (Carlson *et al.*, 2004).

The other important factor for the establishment of ETEC infection is the gastric pH. It has been suggested that incorporating **organic acids** like citric, fumaric, lactic and formic acid might help in reducing the ETEC bacteria and also improve the digestibility of feed. Taube *et al.* (2009) showed that addition of formic acid and propionic acid in the feed reduced the count of experimentally infected *Salmonella* and *E. coli* in piglet gut (Taube *et al.*, 2009). Exploration of organic acids might be an option worth considering, especially since the production of most of these organic acids is inexpensive. The mode of action of organic acid in preventing PWD is probably due to the direct action of acids on the ETEC bacterium. The acids are capable of defusing into the bacterium. This would have lethal consequences on the ETEC bacterium, denaturing the essential enzymes, jeopardising the integrity of purine bases and dramatically altering the turgor pressure in the cell (Heo *et al.*, 2012; Warnecke and Gill, 2005).

Probiotic and prebiotic approaches have also been experimented with as alternative safe prophylaxis against PWD. However there are no general

consensuses in the use of probiotics yet. It is believed that a more regulated dosage and temporal feeding regimen has to be streamlined to attain high consistent efficacy (Bontempo *et al.*, 2006). For instance, administration of *Lactobacillus plantarum* once at weaning, at a dose of 3 x 10^9 resulted in significant reduction of challenged ETEC (O149: K91: F4ac). The authors conclude that this dose and treatment regimen leads to better results than other combination of regimen and doses evaluated (when higher dose of 5 x 10^9 or when *L plantarum* was administrated 3 days before weaning). In another example Setia and co-workers demonstrated the inhibitory activity of colicin producing *E. coli* in inhibiting ETEC *in vitro* (Setia *et al.*, 2009). Along with secretion of antimicrobial substances like bacteriocin or pH altering organic acids and short fatty acids, it is suggested that some probiotics themselves inhibit the ETEC adherence by steric hindrance and competitive exclusion (Heo *et al.*, 2012; Roselli *et al.*, 2005). *Enterococcus faecium* in an *in vivo* assay and *Lactobacillus fermentum* strains 104R in an *in vitro* system have shown to exhibit such inhibitory effect against ETEC, the exact mechanism of their action though is unknown (Blomberg *et al.*, 1993; Jin *et al.*, 2000).

As prebiotics, the use of non-starch polysaccharide hydrolysis products from wheat and flax (Kiarie *et al.*, 2010) and in another study with hydrolysis products from soybean and canola (Kiarie *et al.*, 2008) have showed to protect against ETEC infection related fluid loss, by segment perfusion test. In an *in vivo* ETEC challenge experiment the use of raw potato starch in feed had positive effect on faecal consistency, comparable to the results obtained on feeding antibiotic bearing feed (Bhandari *et al.*, 2009). Some researchers in the field of probiotics and prebiotics also suggest the a synergistic effect might be obtained by combination of the two principles in feed (Heo *et al.*, 2012).

The diet, particularly the protein content influences the digestibility and nutrition; thus the susceptibility of ETEC and progression of PWD. Over the years there has been different feed formulation tested for better growth and with additive effect on maintain gastric physiology to preventing ETEC infection. Proteins in the feed are sourced from plants as well as animals. Some of these plant proteins might also have specific inhibitory function. Becker *et al*. (2011),

demonstrated in an *in vitro* inhibition assay that the pea hulls and faba bean hulls impaired the F4⁺ETEC interaction with receptors and their LT enterotoxin's binding to the GM1 ganglioside receptor (Becker *et al.*, 2011). However overall, it is seen that animal proteins are much better digested than plant protein, this could be due to the anti-nutritive factors in many protein rich plant sources like soybean (Heo *et al.*, 2012). The effect of better digestibility and nutrition are immediately reflected in better weight gain, and also in preventing the severity of ETEC infection (Owusu-Asiedu *et al.*, 2002b; Sola-Oriol *et al.*, 2011). A positive effect of spray-dried animal plasma-based diet is probably due to the **anti-ETEC antibodies in the plasma fraction** (Owusu-Asiedu *et al.*, 2002a). Similar feed based passive protection can be achieved with spray-dried **egg yolk anti-ETEC antibodies** from immunised hens (Vila *et al.*, 2010; Yokoyama *et al.*, 1992). However there are concerns about the use of animal plasma due to fear of introducing pathogens (like viruses and prions), while processing the animal protein and autoclaving them before feeding does not render similar efficacy (Owusu-Asiedu *et al.*, 2002a). The egg yolk antibodies on the other hand are comparatively safe but are very expensive, at the cost of EUR 65/kg of egg yolk protein (Chernysheva *et al.*, 2004). Harmsen *et al.* (2005) suggested cost effective production of **anti-ETEC antibodies in yeast expression system** for oral passive immunisation and produced anti-FaeG, antigen binding variable domains of a heavy chain only lama antibody (VHH) (Harmsen *et al.*, 2005). Though the *in vitro* segment perfusion tests results of these anti-FaeG VHHs were promising, piglets when fed with this VHH producing yeast broth failed to protect *in vivo* on F4⁺ETEC challenge. The proteolysis in gastric environment or the monovalency of VHH could be the reasons for failure to prevent ETEC infection and diarrhoea (Harmsen *et al.*, 2005). On the same lines of cost effective production, a company called Novoplant (Germany) developed **anti-F4 single chain antibodies called, BA11 in pea seeds** (Saalbach *et al.*, 2007). Production of antibodies in seeds and its retention in the (seed) endomembrane protects the plant made antibodies from the harsh gastric environment (Zimmermann *et al.*, 2009). Perhaps due to such bio-encapsulation, Novoplant's in pea made orally delivered BA11 effectively protected the piglets from F4⁺ETEC challenge. Sadly, in 2008 Novoplant filed for bankruptcy and seemingly since then the

development of BA11 has ceased.

Apart from passive immunisation, **oral active vaccination** has also been investigated. To prevent the ETEC bacteria from attaching, colonising and thus establishing infection a local mucosal immunity is needed (Fairbrother *et al.*, 2005). Intramuscular vaccination leads to good systemic immune response but does not elicit mucosal immunity (Van den Broeck *et al.*, 1999a; Van den Broeck *et al.*, 1999b; Van der Stede *et al.*, 2003). Oral vaccination with either attenuated ETEC strain or with purified fimbriae in contrast to needle-based vaccination gives rise to a significant mucosal IgA and IgM response (Snoeck *et al.*, 2003; Van den Broeck *et al.*, 1999a).

However there are two hurdles in oral vaccination. In order to have immune response at weaning, the piglets need to be immunised at least one week prior, i.e. when they are suckling. However the maternal anti-ETEC antibodies in milk present a risk of neutralise the vaccine fimbriae or the attenuated strain before it can stimulate the immune system (Fairbrother *et al.*, 2005). Secondly the gastric pH— it has been demonstrated by *in vitro* simulations in gastric fluid that purified fimbriae get completely digested at pH 2 by 3 hours (Snoeck *et al.*, 2004). In this regard enteric-coated pellets or encapsulated, or fimbriae in tablet format might be better protected in the gastric juices (Calinescu *et al.*, 2005; Huyghebaert *et al.*, 2005; Snoeck *et al.*, 2003). Snoeck et *al.* (2003) evaluated the efficacy of such **orally delivered enteric-coated F4 fimbriae** in challenge experiment; though a clear mucosal and systemic immune response was achieved the treatment did not protect against ETEC colonisation, and only a marginal significant reduction in $F4^+$ETEC shedding titer was observed. As a cost effect mass production system for **encapsulated vaccine development**, the FaeG antigen was expressed in alfalfa, tobacco and barley (Joensuu *et al.*, 2004; Joensuu *et al.*, 2006a; Joensuu *et al.*, 2006b). The plant made FaeG molecules were functional *in vitro* and led to production of neutralising antibodies in mice. Of these, the alfalfa produced FaeG when fed to piglets along with cholera toxin reduced the shedding of challenged $F4^+$ETEC strain (Joensuu *et al.*, 2006b). The general methods and strategies used for $F4^+$ETEC vaccination should work for $F18^+$ETEC as well (Fairbrother *et al.*, 2005). However for each of the three

strains of F4$^+$ETEC and the F18$^+$ETEC, specific vaccine might be needed, as the cross protection may not be sufficient i.e. antiserum raised due to F4ac vaccination might not protect efficiently against F4ab or F4ad bearing strains, like wise for F18ab and F18ac (Bertschinger *et al.*, 2000; Fairbrother *et al.*, 2005).

Perhaps in near future a cost effective, safe and successful prophylaxis would be available. As compare to ETEC vaccines, which run the risk of being neutralised by maternal antibodies, passive immunisation with anti-ETEC antibodies has had overall better results for ETEC prophylaxis. Plant production systems have the potential of producing large quantities of therapeutic antigen and antibodies at a cost effective scale. However the potential of plant for veterinary therapeutic development is largely unrealised. Perhaps such protective antibodies produced in plants, particularly in edible tissue shall provide for the much-needed solution to prevent ETEC related PWD.

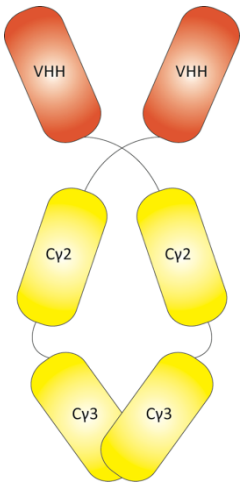
Chapter 4

Seed produced, porcinised lama fusion antibodies prevent enterotoxigenic *Escherichia coli* binding to gut villous enterocytes *in vitro*

Designing a robust antibody format

Vikram Virdi, Sylvie De Buck, Annelies Coddens, Hana Hoffmeisterova, Bruno Goddeeris, Henri De Greve, Eric Cox and Ann Depicker

H.D.G. and A.D. conceived the VHH-IgG fusion strategy. V.V. performed most of the experiment i.e. panning of anti-FaeG VHHs from the FaeG-VHH lama immune library, cloned the VHH-IgG fusion constructs, produced the transgenic Arabidopsis lines expressing VHH-IgGs, standardised the high-throughput functional screening protocol, selected the highest expressing line, and characterised the VHH-IgG antibodies. S.D.B. provided consultation and mentored the project. H.H. helped in screening of the primary transformants. A.C. performed the *in vitro* villous adhesion test. H.D.G. performed the VHH-magnetic bead agglutination assay. V.V. analysed the results and wrote this chapter, while H.D.G and A.D. edited it.



Abstract[9]:

Plants can be used to produce a plethora of recombinant therapeutic proteins at fraction of a cost as compared to conventional production platforms. Plant seeds in particular are natural protein producing and storage organs, which enable production of abundant recombinant protein in stable confined environment. The seed-based expression system is particularly interesting for oral passive immunisation against veterinary diseases, such as enterotoxigenic *Escherichia coli* (ETEC) causing post-weaning diarrhoea. We therefore investigated the production of anti-ETEC antibodies in seeds of the model plant *Arabidopsis thaliana*, to later transfer the technology to a grain crop that can be used in piglet starter feed and produced in sufficient quantities.

In the pilot phase, we focused on the F4 fimbriae bearing ETEC ($F4^+$ETEC) strains that are most predominantly isolated from diseased piglets. For successful seed-based anti-$F4^+$ETEC passive immunotherapy it is imperative that the antibodies are produced in abundant amounts and that they survive gut transit. We hence aimed at designing a robust antibody by fusing the antigen binding domains of camelid antibodies (VHH) with the fragment crystallisable (Fc) region of porcine IgG3. The anti-$F4^+$ETEC VHHs were panned from a lymphocytic library derived from a lama immunised with purified FaeGac adhesin, building up the F4 fimbriae. Four such VHH's were selected from the three clusters representing the total diversity of the library. The VHHs were grafted to the Fc, then inserted into a seed specific expression cassette and transformed in *A. thaliana*. Each antibody expressed to its own particular accumulation level, the highest being 3% of seed weight.

These highly expressed anti-$F4^+$ETEC porcinised-camelid antibodies were correctly folded *in planta* and recognised all the serotypes of the FaeG adhesins. The disulphide bond within the hinge enabled production of dimeric antibodies that

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doi: 10.3389/conf.fimmu.2012.01.00008

agglutinate the F4+ETEC bacteria. Moreover the crude seed extract bearing antibodies inhibited F4+ETEC attachment to gut villous enterocytes in an *in vitro* assay, making the seed mix very interesting to evaluate protection in a piglet challenge experiment

Introduction

Plants have been used to produce a plethora of different recombinant proteins (Paul and Ma, 2011; Xu *et al.*, 2011a). Over the last two decades there has been tremendous improvement in the plant expression strategies, leading to high level expression of complex proteins (Rybicki, 2010; Streatfield, 2007), even with engineered glycans (Loos *et al.*, 2011a; Saint-Jore-Dupas *et al.*, 2007), enabling plants to produce not just bio-similar products but superior quality products with enhanced features (Faye and Gomord, 2010). Plants can effectively produce complex proteins like assembled secretory IgA (Ma *et al.*, 1995) and complex virus like particles such as the blue tongue virus particles[10] (Thuenemann and Lomonossoff, 2010). Production of molecules like these would be very difficult via mammalian or other conventional expression systems, and far from being a commercially viable option (Paul and Ma, 2011), thus plant expression systems are carving their own niche among other protein production systems. Together with this flexibility, it is the cost-effective scalability of plant-made proteins that makes them such an attractive platform (Obembe *et al.*, 2011; Xu *et al.*, 2011a).

Production platforms that have the capacity of providing globally affordable therapeutic proteins are imperative for combating human diseases, but such promising platforms also provide opportunities for developing veterinary therapeutic proteins. The cost of production for a marketable veterinary therapeutic protein has to be significantly low, particularly for farm animals since large numbers of animals need to be treated often at fairly regular interval of time. Thus the net capacity to manufacture a veterinary therapeutic protein needs to be very high without adding to the cost per dose. Cost effective plant production systems have

[10] Conference abstract book available at http://goo.gl/z7MdS

given an impetus to development of new and orphan veterinarian pharmaceuticals (Rybicki, 2010), however their full potential for producing therapeutics against animal diseases is still to be realised. Moreover, oral immunisation (passive as well as active) can be achieved by direct in-feed production of therapeutics. This in-feed oral delivery system adds to the ease of administration, limiting the necessity of trained personnel for administering the therapeutics (Boothe *et al.*, 2010; Streatfield, 2006). In-feed, oral immunisation with antigen or oral passive immunisation with antibodies is particularly advantageous for developing treatments against enteric disease like enterotoxigenic *Escherichia coli* (ETEC) related post-weaning diarrhoea (PWD) in piglets; as it is can provide local mucosal immunity (intestinal) against ETEC, which is needed for prevention of PWD (Bianchi *et al.*, 1996; Moon and Bunn, 1993; Van den Broeck *et al.*, 1999b; Verdonck *et al.*, 2004b).

Quite a few strategies have been experimented with to provide protection against ETEC at the intestinal surface; this is primarily because of the disease burden. ETEC related PWD leads to heavy economic losses to the global porcine rearing industry (Amezcua *et al.*, 2002; Fairbrother *et al.*, 2005; Hong *et al.*, 2006). Moreover, the urge to find prophylactic measures is increasing because of the increasing ban on the traditionally used antibiotic prophylaxis (Berghman *et al.*, 2005). Of the different ETEC strains, the ones bearing the F4 fimbriae ($F4^+$ETEC) are predominantly isolated from piglets with acute diarrhoea (Osek, 1999). However, currently there are no commercially available specific therapeutics against $F4^+$ETEC and until very recently, antibiotics were the only treatment extensively utilised in several parts of the world (Fairbrother *et al.*, 2005). Attempts so far to develop suitable anti-$F4^+$ETEC therapeutics have broadly focused on three strategies, first- vaccine development, including plant based vaccine (Joensuu *et al.*, 2004; Joensuu *et al.*, 2006a; Joensuu *et al.*, 2006b); second- passive immunisation with spray-dried animal plasma or immunised egg protein (Chernysheva *et al.*, 2004; Marquardt *et al.*, 1999; Yokoyama *et al.*, 1992), and third the use of natural ETEC inhibitors in piglet starter feed (Becker *et al.*, 2011; Mynott *et al.*, 1996). Of the three, passive

immunisation strategies have fared relatively well, as passive immunisation provides instant protection against ETEC. However there are limitations to each of these passive immunisation strategies (Owusu-Asiedu *et al.*, 2002a). Immunisation of the sow induces lactogenic immunity, producing large amount of anti-F4$^+$ETEC antibody in the milk, but this protection is lost soon after weaning. Passive immunisation with serum antibodies obtained from animal plasma is difficult to regulate and be consistent in the content of anti-F4$^+$ETEC antibody. Immunised egg antibodies though have the merits of regulating and maintaining consistency; the cost of such therapy is very high which makes it an economically unrealistic treatment (Chernysheva *et al.*, 2004; Harmsen *et al.*, 2005).

We explored an economic viable solution to produce specific antibodies against ETEC, by producing them in seeds that can be incorporated into the starter feed of weaned piglets. Production of anti-ETEC antibodies in seeds also has the merits of ease of storage and obtaining high concentrations in confined space (Lau and Sun, 2009; Van Droogenbroeck *et al.*, 2007), which further adds to the ease of administration, particularly for large herd of piglets. However oral passive immunisation has two important pit falls, first the harsh acid environment of the gastrointestinal tract and the necessity of high amounts of antibody, usually being milligrams of antibody per individual per day (Streatfield, 2006).

To circumnavigate these two issues, we aimed at designing a robust but simple antibody, based on the camelid heavy chain only antibody structure. The camelids have a unique kind of antibodies that are devoid of light chain and the light chain assorted (first) constant heavy domain one (CH1). These heavy chain antibodies have a variable antigen binding domain (VHH), nick named Nanobody®, followed by a hinge and the CH2-CH3 domains (Harmsen and De Haard, 2007). The VHHs are very robust and have been described to survive harsh chemical and temperature conditions and still remain functional (Muyldermans, 2001). It is this property of VHH that makes them particularly attractive for designing anti-F4$^+$ETEC antibodies for oral passive immunisation therapy. Monomeric VHHs against the F4 fimbriae of pathogenic ETEC have been shown to prevent ETEC binding to gut villous

enterocytes and even reduce the intestinal fluid loss, *in vitro* (Harmsen *et al.*, 2005). However agglutination of ETEC bacteria by bivalent antibodies prevents biofilm formation by cross-linking ETEC and is a crucial element in preventing diarrhoea.

VHHs can be made bivalent by fusing them to the fragment crystallisable (Fc) region of immunoglobulins. These bivalent VHH-Fc fusions have an increased half life and also an increased avidity (Hmila *et al.*, 2008). Additionally, Fc as a fusion tag has been shown to boost the expression of several heterologous proteins in seeds (Sylvie De Buck[11] and Robin Piron[12], personal communication) and in leaves (Obregon *et al.*, 2006). The choice of a specific Fc can further impart important attributes required for protective immunity. There are 6 putative subclasses of the porcine IgG antibodies. Of these, the porcine IgG3 has the longest hinge with additional cysteine residues. From the sequence analysis, the porcine IgG3 has been predicted to be most likely to activate the complement and bind to the Fcγ receptors. And most importantly, porcine IgG3 might also be relatively insensitive to pepsin cleavage (Butler *et al.*, 2009). These properties suggested to us that the Fc fragment of porcine IgG3 might be an ideal fusion partner for ETEC binding VHHs to be used for oral passive immunotherapy.

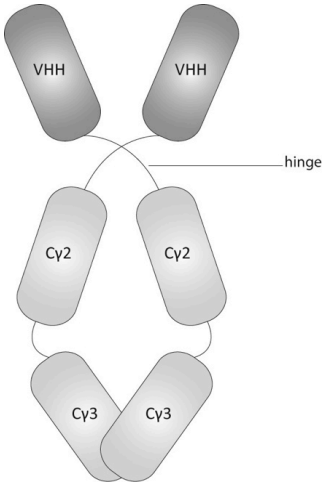


Figure 4.1: Schematic diagram of the customised porcinised-lama antibody, consisting of VHHs (anti-FaeGac) grafted on to the long flexible hinge (23 amino acid), constant heavy domain 2 (Cγ2) and constant heavy domain 3 (Cγ3) of the porcine IgG3.

[11] VHH grafted to human IgG Fc
[12] Viral capsid protein in fusion to porcine Fc

With this rational, 4 anti-F4+ETEC VHH domains were isolated, engineered into customised VHH-IgG3 'porcinised-lama' (PoLa) antibodies (Figure 4.1) and produced in the seeds of the model dicot plant *Arabidopsis thaliana* (ecotype- Col 0), to deliver a proof of feasibility of in seed expression. Here we describe the PoLa antibody expression results and present evidence of their functionality in preventing F4+ETEC attachment to villous enterocytes *in vitro*.

Results and Discussion

Selection of functional VHHs that agglutinate all three serotypes of F4+ETEC bacteria in multivalent form

For isolating robust antigen binding domains, suitable for oral passive immunotherapy, VHH's were panned against FaeG, the major adhesin protein present on the proteinaceous fimbriae of F4+ETEC. Attachment of FaeG to the porcine gut villous enterocyte receptors is the first step in establishing ETEC infection, followed by colonisation, toxin release and the consequential diarrhoea. Specific VHHs that inhibit bacterial binding can in principle prevent the onset of diarrhoea (Harmsen *et al.*, 2005). Serologically the FaeG adhesin can be classified as: FaeGab, FaeGac and FaeGad depending upon the variable epitope of the adhesion molecule FaeG (see review by Van den Broeck *et al.*, 2000). Of these three variants, predominantly isolated F4+ETEC bearing FaeGac adhesin on the fimbriae, has often been associated with acute diarrhoea in piglets (Gonzalez *et al.*, 1995). Purified recombinant FaeGac was used to immunise a healthy lama to develop FaeG-specific VHHs against this major adhesin. Six weeks post immunisation, the lymphocytic cDNA immune library was made. Using phage display technology, the VHHs from the immune library were panned against the antigen FaeGac and FaeGad for isolating VHHs specific for the variable epitope ('c' of predominantly occurring F4+ETEC FaeGac) as well as against the conserved epitope ('a') common to all 3 serotypes of F4+ETEC. This enabled the selection of a panel of 79 VHHs recognising specifically the FaeGac along with broad spectrum VHHs recognising all the three variants of FaeG. These VHHs specifically recognised FaeG as well as purified F4 Fimbriae in an enzyme-linked immunosorbent assay (ELISA). To eliminate similar, redundant

VHHs, we used the restriction fragment length polymorphism (RFLP) technique. Whereby, the VHH coding sequence was amplified by PCR using specific primers, which resulted in a 680 bp amplicon. This PCR product was then digested with restriction enzyme HinfI or RsaI and the pattern of digested fragments was visualised by agarose gel electrophoreses. VHHs representing similar patterns were clubbed into RFLP groups and, the DNA of representative VHHs from each of these RFLP groups were sequenced.

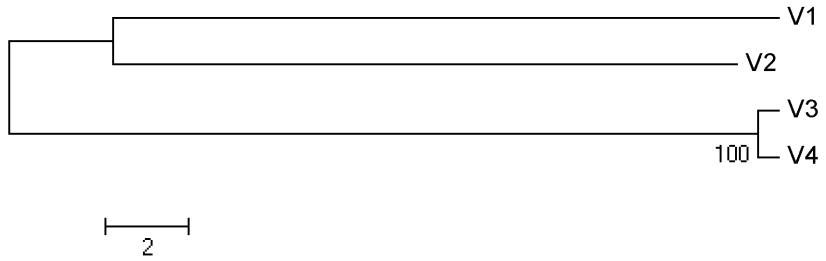


Figure 4.2: Amino acid sequence divergence between the four anti FaeGac VHHs. The neighbour joining, 1000 replicates bootstrapped tree represents the three clades of VHHs isolated. The scale bar indicates the number of difference in amino acid composition. The boot strap value (100) is denoted at the node.

```
                FWR1                    CDR1          FWR2          CDR2      FWR3
V1  QVQLQESGGG LVQAGGSLRL SCEASGNVDR IDAMGWFRQA PGKQREFVGY ISEGGILN-Y  [ 59]
V2  .......... ...P...... ..T...SISS .N....Y... ..SK....AH .TNT.VTE-F  [ 59]
V3  .......... .......... ..A...LTFD TY........ ...K..Y.AA ..WT..STY.  [ 60]
V4  .......... .......... ..A...LTFD TY........ ...K..Y.AA ..WT..STY.  [ 60]

                FWR3                          CDR3          FWR4
V1  GDFVKGRFTI SRDNAKNTVY LQMSNLKSED TGVYFCAASH WGTLLIKGIE HWGKGTQVTV  [119]
V2  A.S....... ......T..D ...NS..P.. .A..Y...TD .........D ..........  [119]
V3  A.IA...... ........L. ...DS..P.. .A..Y...Q[K] SLNVPAP-WD Y..Q......  [119]
V4  A.IA...... ........L. ...DS..P.. .A..Y...Q[R] SLNVPAP-WD Y..Q......  [119]

    FWR4
V1  SS [121]
V2  .. [121]
V3  .. [121]
V4  .. [121]
```

Figure 4.3: Amino acid sequence alignment of the 4 selected anti-F4 VHH. The 4 amino acid sequences of VHH V1-4 are compared between one another, the sequence of VHH V1 is at the top of this alignment, in reference to which amino acid changes in the other three VHH sequences are indicated; identical amino acid residues are represented with a dot. The 4 framework regions (FWR) interspersed with the 3 complement determining regions (CDRs) are indicated above the alignment. The single amino acid difference Lys-100-Arg between the VHH sequence of V3 and V4 in the CDR3 region is bold face. Reference to amino acid position is indicated in parenthesis at the end of each line.

A typical sequence of a VHH shows structurally conserved regions in which three distinct hypervariable regions are embedded called complementarity determining regions (CDRs) (Muyldermans *et al.*, 2001). Of the three CDRs, the CDR3 loop is regarded most important in interaction with the antigen. Often the CDR3 forms a protrusion that interacts with deep clefts of antigen (Transue *et al.*, 1998). It is this convex structure, unique to the VHH, that together with the CDR1 provides the VHH with an enhanced repertoire of antigen binding paratopes (Muyldermans *et al.*, 2001). From the deduced amino acid sequence and the divergence in CDR3, all isolated 79 VHH sequences could be divided into three clades. Four VHHs representing these three clades were selected and named V1, V2, V3 and V4 (Figure 4.2). The VHHs V3 and V4 belonged to the third clade and differed by a single amino acid substitution Lys-100-Arg within the CDR3 loop (Figure 4.2 and 4.3).

To investigate the interaction of VHHs with F4$^+$ETEC bacteria, the simple bacterial agglutination test was used. Bacterial agglutination test is a standard serological test, where presence of antibody or antigenic variant can be determined by visualising the agglutination formed on a glass slide by cross-linking bivalent antibody. Since VHHs themselves are monovalent and incapable of crosslinking bacteria, purified VHHs were covalently linked to magnetic beads making them multivalent. The dark colour of these magnetic beads also helps in visualizing the results of the agglutination, as compared to colourless resin beads.

The anti-FaeGac VHH-coated magnetic beads with each of the 4 VHHs when incubated with different serotypes of F4$^+$ETEC bacteria showed agglutination. On the contrary, the same beads did not show agglutination with the negative control *E. coli* strain K514 (Colson *et al.*, 1965; Johansen *et al.*, 1999), which does not produce any fimbriae nor with K514 transformed with plasmid pIH120 expressing F18 fimbriae (K514-pIH120) as non-specific fimbriae control. In Figure 4.4 the results of the agglutination tests are shown for beads coated with V2 and V3.

Thus all 4 VHHs isolated in this study, in multivalent format were able to agglutinate the three variants of F4$^+$ETEC.

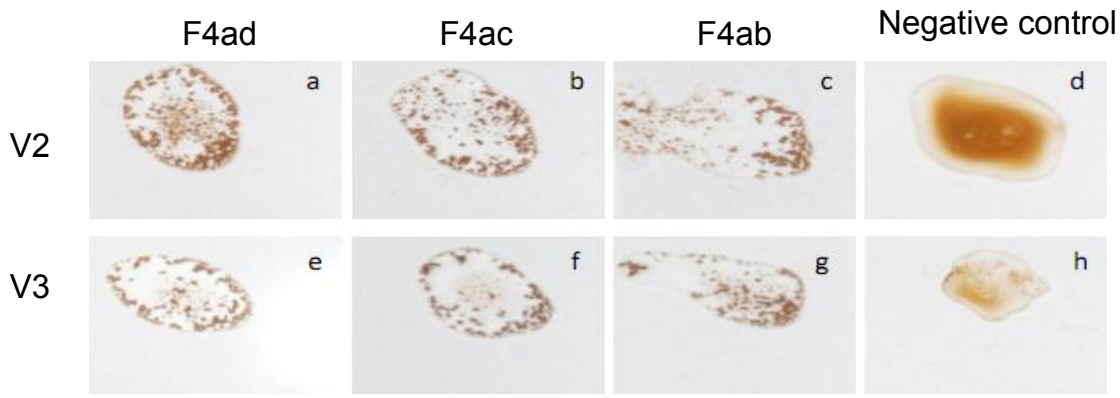


Figure 4.4: Anti-F4⁺ETEC VHH V2 and V3 in a multivalent form coupled to magnetic beads specifically agglutinates all the 3 serotypes of F4⁺ETEC. Bead coated VHH V2 and V3 show specific agglutination when incubated with F4⁺ETEC strain C585-80 expressing F4ad fimbriae (a and e respectively), F4⁺ETEC strain C95-72 expressing F4ac (b and f respectively) and F4⁺ETEC strain C1023-78 expressing F4ab (c and g respectively); while not with negative control strain K514-PIH120 (d and h). (Similar results obtained with VHH V1 and V4 are not shown.) The interaction of anti-ETEC VHHs was specific and no agglutination was observed with an unrelated VHH (Nb226) used as control in the above test (not shown in the figure). Figure adapted from Master thesis (Okello, 2010).

Production of high accumulating porcinised-lama divalent antibodies in Arabidopsis seeds

The agglutination potential of the 4 VHHs in multivalent form makes them promising candidates as anti-F4⁺ETEC therapeutic for oral feed based passive immunisation. Hence we further focused on strategies that would enable high production of the envisaged recombinant antibodies in seeds and devised screening methods to identify the high expressing transgenic plants.

Firstly the native sequence of all four anti-F4⁺ETEC monovalent VHHs i.e. V1, V2, V3, and V4 were grafted on the codon optimised sequence of porcine IgG3 Fc to produce divalent porcinised-lama heavy chain only antibodies (PoLa), which were named V1G, V2G, V3G and V4G respectively. The coding regions of these four fusion PoLa antibodies with an N-terminal 2S2 signal peptide of seed storage protein, and a C-terminal endoplasmic retention tag (KDEL) were cloned within the pPhas GW vector (Figure 4.5) (Morandini *et al.*, 2011), bearing the 5' β-Phaseoline promoter and the 3' arceline terminator bearing regulatory sequences to enable dicot seed-specific high expression of heterologous proteins (Loos *et al.*, 2011a; Loos *et al.*, 2011b; Van Droogenbroeck *et al.*, 2007).

Further, by *Agrobacterium*-mediated floral dip transformation all the 4 PoLa antibodies were transformed in *A. thaliana* Col 0. Five plants were dipped per antibody construct, for which a transformation efficiency of 1.3% of T1 seeds was observed on an average. At least 24 selected T1 transformants were grown for each of the PoLa antibodies and T2 seed stocks were harvested, and characterised for the antibody expressed.

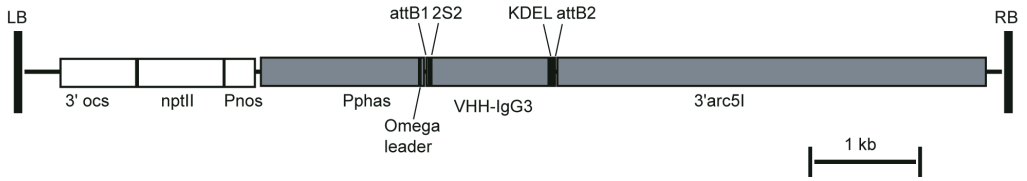


Figure 4.5: Schematic, to scale representation of the T-DNA construct used for in seed expression of anti-F4⁺ETEC porcinised-lama VHH-IgG antibodies. LB- left border; RB- right border; VHH-IgG3-fused coding sequence of the VHH and porcine IgG3 Fc; Omega leader- 5' tobacco mosaic viral UTR; 2S2 - signal peptide sequence of the 2S2 seed storage protein; KDEL- endoplasmic retention motif; attB1,attB2 - gateway recombination site sequences (Invitrogen); Pphas- Phaseolin promoter; 3'arc5I- 3' arcelin regulatory sequence; nptII- neomycin phosphotransferase II gene; Pnos- nopaline synthase gene promoter; 3'ocs- octopine synthase terminator.

To evaluate the relative VHH-IgG antibody accumulation levels in the different T2 seed stocks and to identify the high antibody expressing transformants; a high-throughput functional ELISA was setup with antigen-coated wells (FaeGac). Within the 24 transformants screened for each VHH-IgG antibody, the range for variation for the V2G was the highest followed by V1G, while V3G and V4G antibodies had a relatively low range of variation in accumulation of functional antibody (Figure 4.6). From the ELISA read outs, the relative accumulation of functional antibodies in the respective transformants could be classified as highest, medium and low expressers. The top 5, antibody expressing transformants, along with 2 medium expressing and a low expressing plant for each PoLa antibody were characterised further (Figure 4.6). Under reducing condition all the transgenic seed extracts showed a heterologous band migrating at ~49 kDa, this band was also confirmed as PoLa by immunoblot using the anti-pig IgG specific polyclonal serum (Figure 4.6). Under non-reducing conditions the PoLa antibodies migrated at ~80 kDa, thus confirming that they form perfect dimeric antibodies *in planta* (Figure 4.7). However the.

calculated molecular weight of a single PoLa antibody chain was ~40 kDa, the higher molecular weight could probably be due to posttranslational modifications viz. presence of plant N-linked glycans.

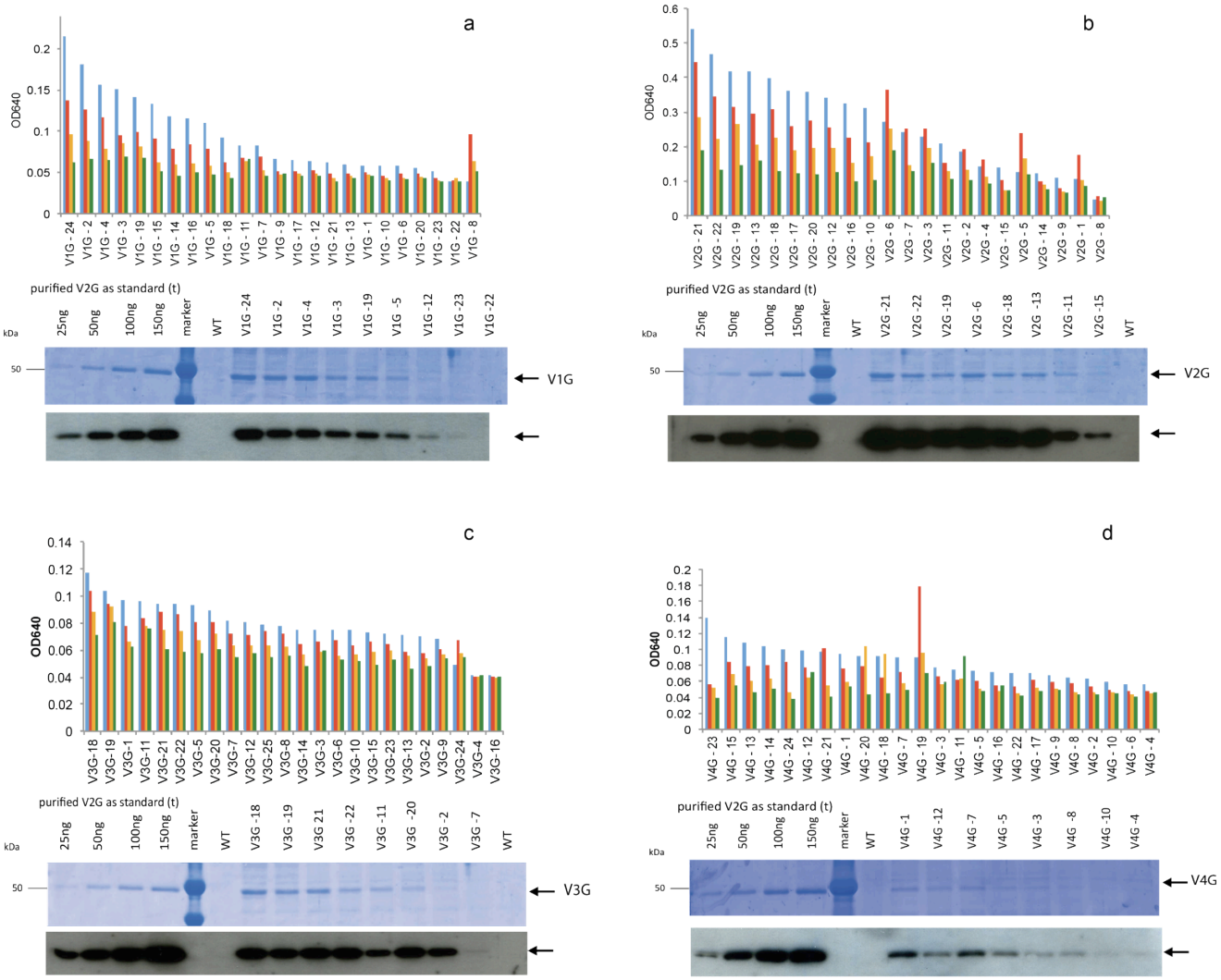


Figure 4.6: Inter transformant variation in accumulation of the 4 different VHH-IgG antibodies. The panel a, b, c and d show results of antibodies V1G, V2G, V3G and V4G, respectively. The graph in each panel shows the functional ELISA OD 405 values of serially diluted 24 T2 seed extracts, as 1:100 (blue), 1:200 (red),1:400 (brownish-yellow) and 1:800 (green); indicative of the comparative functional antibody accumulation levels. Underneath each of the graphs is the corresponding Coomassie stained SDS-PAGE and immunoblot of the seed extracts, the respective transformants are annotated above the get picture. On the right hand side of the marker are wild type (WT) seed extract, followed by the seed extracts from the 5 top antibody expressing plants according to the functional ELISA graph have been loaded in descending order, followed by two medium expressers and one low expressing line. One microgram of total soluble seed protein was added in each well, the antibody bands are indicated with an arrow (~49 kDa). These VHH-IgG bands were quantified using purified standards as indicated (left had side of marker). (t- the purified V2G antibody produced transiently in *Nicotiana benthamiana* leaves was used as standard reference).

The relative accumulation of this 49 kDa VHH-IgG antibody chain was in affirmation with the functional ELISA results, thus showing that the most of the correct molecular weight protein was also functional against FaeGac (Figure 4.6)Further, the 4 VHH-IgGs in divalent format also recognise the other two variants of FaeG, i.e. FaeGab and FaeGad in a similar ELISA setup, reiterating previous broad-spectrum interaction results with VHH coated on magnetic beads (Figures 4.4 and 4.10)

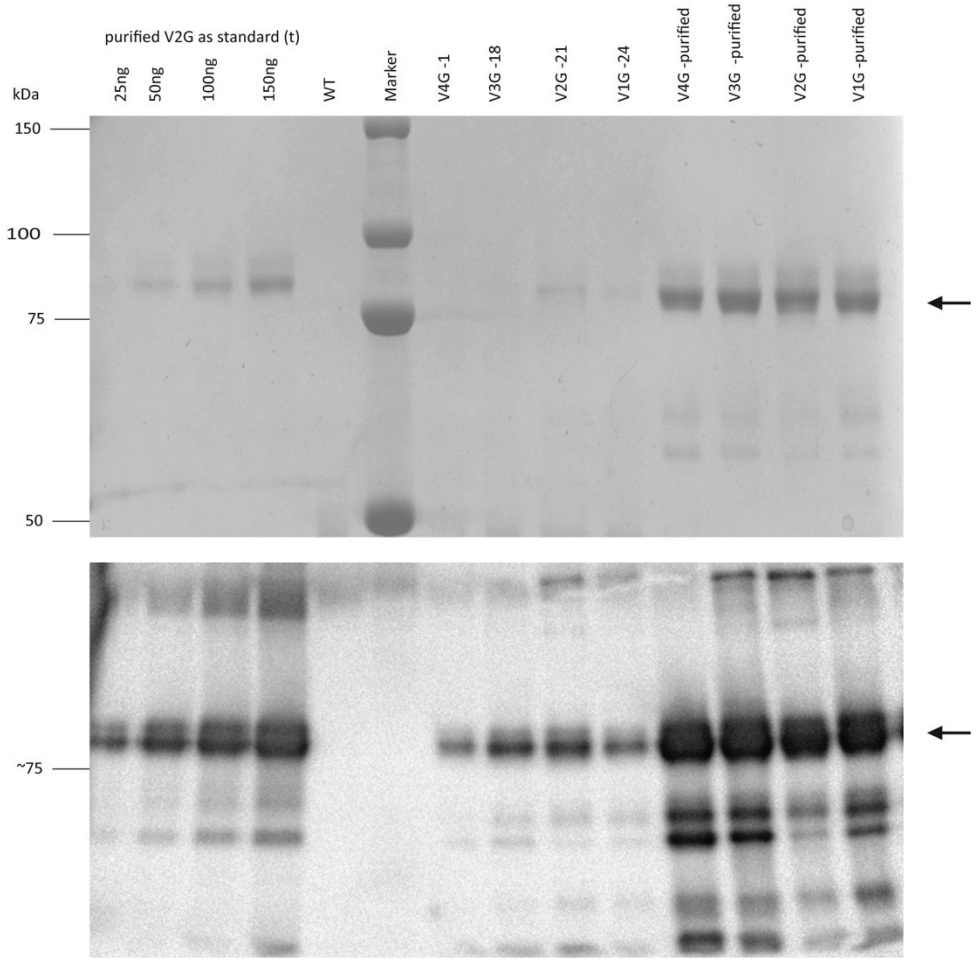


Figure 4.7: All 4 VHH-IgGs form dimeric antibodies in seeds. Purified (500 ng/well) VHH-IgG as well as seed extract (1 µg of total soluble protein in each well) from lines V1G-24, V2G-21, V3G-18 and V4G-1 expressing high amounts of antibody were separated on 7% SDS-PAGE under non-reducing conditions, and stained with Coomassie (upper panel) or immunoblotted (lower panel) and developed with polyclonal anti-porcine IgG serum. The arrows indicate the high molecular weight dimeric VHH-IgG (~80 kDa). All the lanes and the reference molecular weight bands are annotated in the image; WT indicates wild type Arabidopsis seed extract (t- purified standard produced in *N. benthamiana* leaves).

Using purified V2G produced in *Nicotiana benthamiana* leaves as standard reference, the accumulation of the different antibodies was quantified. V2G was noted as the highest expressing PoLa antibody construct (15% of TSP i.e. total soluble protein, Figure 4.6a) closely followed by V1G (10 % of TSP, Figure 4.6b), where as the antibodies V3G and V4G which differed by a single amino acid, belonging to the same clade (Figures 4.2 and 4.3) expressed to identical levels (about 2 % of TSP each, Figure 4.6, c and d) which was 7 to 5 times lower than the other two PoLa antibodies.

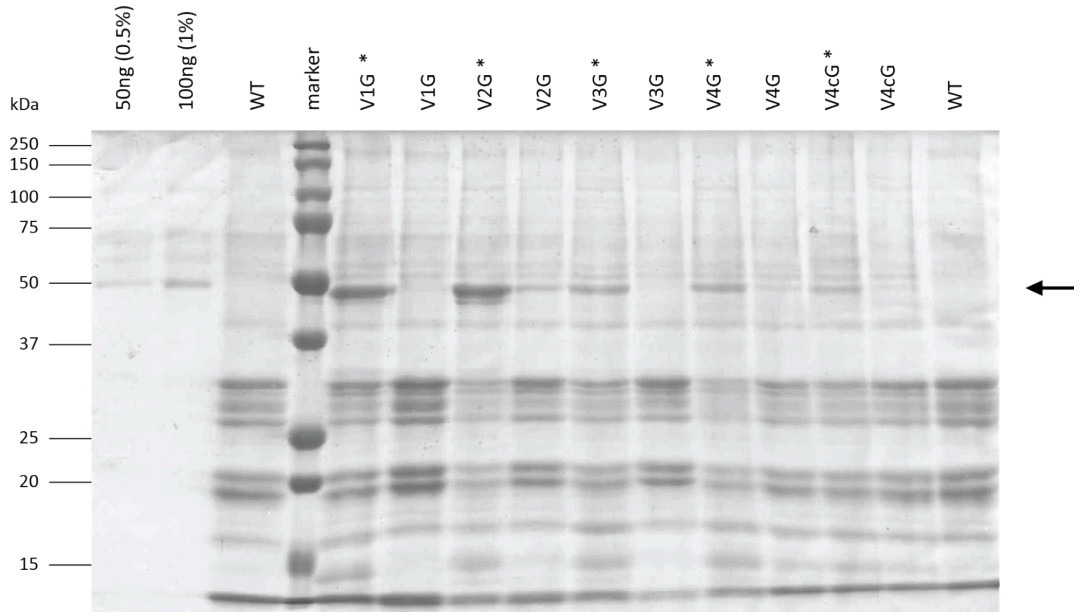


Figure 4.8: Comparison of antibody accumulation levels. Seed protein extracts from the highest (*) and the lowest accumulating Arabidopsis transformants (alternatively placed after marker) for each respective antibody construct (the antibody type is indicated above the lane) were separated on a 12% SDS-PAGE and stained with Coomassie blue. WT stands for wild type untransformed Col 0 seed extract, while 'marker' indicates the precision plus® dual colour protein ladder (Bio-Rad). Ten micrograms of seed extract has been loaded in each well, except first two lanes, where 50 ng and 100 ng of purified antibody V2G has been loaded as standards for quantification, which for the given gel would represent intensity of 0.5% and 1% accumulation of TSP respectively. The arrow indicates the ~49 kDa antibody band within the seed proteins. Codon optimised PoLa antibody V1cG has not been loaded on this gel.

Highest Antibody Accumulation

	proportion of total soluble protein	proportion of seed weight
V1G	10%	2%
V2G	15%	3%
V3G	2%	0.4%
V4G	2%	0.4%
V1cG	10%	2%
V4cG	2%	0.4%

The 4 different PoLa antibodies only differing in the amino-terminal VHH domain showed difference in their maximum protein accumulation levels, ranging from 3% of seed weight (V2G) to 0.4% of seed weight (V3G and V4G) (Figure 4.8). This variability might be contributed by several factors, which for the sake of discussion can be broadly classified as pre-translation and post-translation factors or events. Given that the T-DNA regulatory elements within the expression cassette used were identical, and that for each PoLa antibody 24 different lines were screened, normalizes for the position effect and copy number influence in variation. The variability observed here, thus is most likely due to translational or posttranslational influences.

Along with the regulatory elements, codon optimisation is another factor that is often advocated for attaining higher protein expression (Geyer *et al.*, 2010). Initially, within all the 4 PoLa antibodies, only the coding sequence of the identical porcine Fc region (~ 2/3rd of the PoLa coding sequence) was optimised for Arabidopsis seed codon usage. To evaluate if higher accumulation can be attained by further additional codon optimising of also the VHH domain, the lama codon usage within the VHH coding frame was also optimised to Arabidopsis seed codon usage. Native VHH codons of high expressing PoLa antibody V1G and low expressing V4G were thus exchanged with the codon preferences of Arabidopsis seed storage proteins. However this did not result in any change in the protein accumulation: the codon optimised version V1cG (of V1G) showed 10% TSP accumulation while V4cG (optimised V4G) accumulated to 2% of TSP, each similar to its unoptimised counterparts (Figure 4.8 and 4.9). Thus as per our observation, codon optimisation and the introduced changes in the transcript sequence did not affect the accumulation of the 2 PoLa antibodies. In all, our results from codon optimisation reiterate Ed Rybicky's conclusion drawn from observations in his group and of others that, it is naive to assume plant codon usage to be always optimal (Rybicki, 2010). Rather, a high GC content typically seen in human codon usage is suggested

to influence accumulation of heterologous proteins in plants (Biemelt *et al.*, 2003)[13]. However the GC content in all of the 4 PoLa transcripts is higher than 50% and reasonably similar (52.5% for V1G, 53.7% for V2G, 53.6% for V3G, 53.8% for V4G, 53.5% for V1cG and 53.9% for V4cG). Thus also the GC content in our case does not explain the observed inter antibody variation.

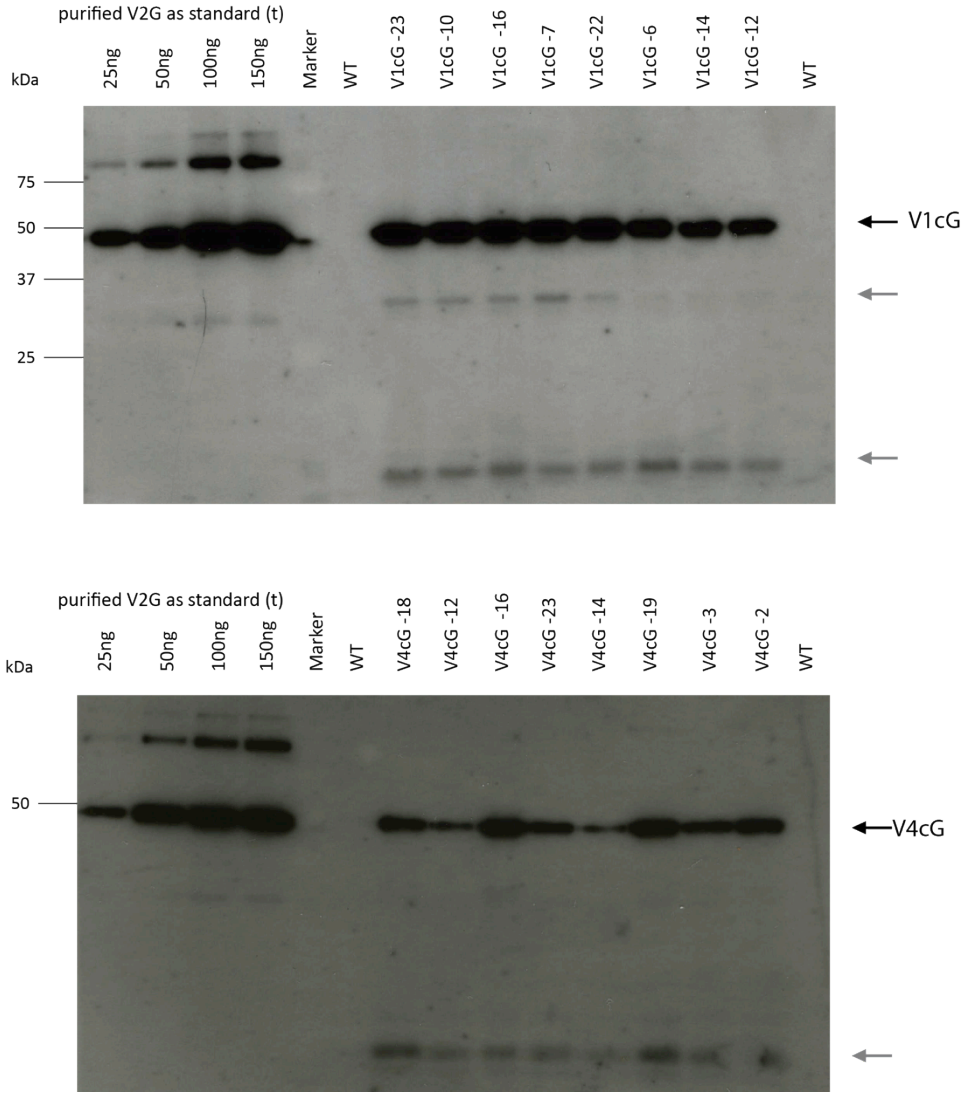


Figure 4.9: VHH codon optimisation did not affect the accumulation of respective antibodies. The above two immunoblots show accumulation levels of the top 5, two medium, and a low expressing line among the 24 lines screened by ELISA; of the V1cG bearing codon optimised version of V1, (compare accumulation with V1G), in top panel and that of V4cG, with codon optimised version of V4 (compare accumulation with V4G) bottom panel are sequentially loaded. The names of the transformants have been annotated in the image, where the black arrow points to the respective

[13] Discussed by Catherine Pineo at the Plant based Vaccine and Antigen conference, Porto, 2011. Expression and optimisation of human papillomavirus type 16 (HPV-16) chimaera candidate vaccines in *Nicotiana benthamiana*' Catherine Pineo, Inga I Hitzeroth, Gillian de Villiers and Edward P. Rybicki.

antibody revealed by a polyclonal anti-porcine IgG serum. The small grey arrows indicate the *in seed* proteolytically cleaved fragment (NB: This proteolytic fragment was seen in all VHH-IgG).

Often modulation/ substitution of certain amino acid residues of the heterologous protein improves the assembly and accumulation of heterologous protein in plants (Waheed *et al.*, 2011), since it might influence the protein stability and thus the accumulation in a given tissue. The sequence of the 4 PoLa antibodies expressed in the Arabidopsis embryo is identical in the Fc region, and variable exclusively in the VHH domain. Thus inferring that the amino acid sequence of the VHH might influence the VHH-Fc accumulation in seeds, and different VHH sequence leads to variability in expression.

However lama VHHs show up to 94% identity, with high similarity within the structural framework regions, and the variability is rather limited to the three hypervariable CDRs; the same was also observed within the 4 anti-ETEC VHHs in this study (Figure 4.3). The V3G differs from V4G by one amino acid in the CRD3 region; the V3G is 37 amino acids different from the V2G, and 30 amino acids different from V1G (Figure 4.3). From these results it appears that not just the whole protein composition but especially the amino acid composition towards the N-terminal end of the protein (here the VHH) influences its accumulation. Arguably, this could just be an isolated case of the VHH-IgG in this study, where average accumulation pattern for 24 transformants of V1cG and V1G was similar (highest 10% of TSP) and so was true for V3G, V4G, and V4cG (highest 2% of TSP).

VHHs when expressed in *E. coli* cells by themselves as monomers also show variability in production, and also differ in their physical properties. In order to build chimeric VHH with high stability and production levels, Saerens and co-workers (2005) identified a universal VHH framework. Grafting the antigen binding loop onto this identified framework led to improved accumulation in *E. coli* and increased thermodynamic stability compared to the original VHH domain (Saerens *et al.*, 2005). Further investigation, in the N-end rule of seeds and by determining VHH frame work suitable for in-seed production will help in designing VHH-Fc which might have higher accumulation.

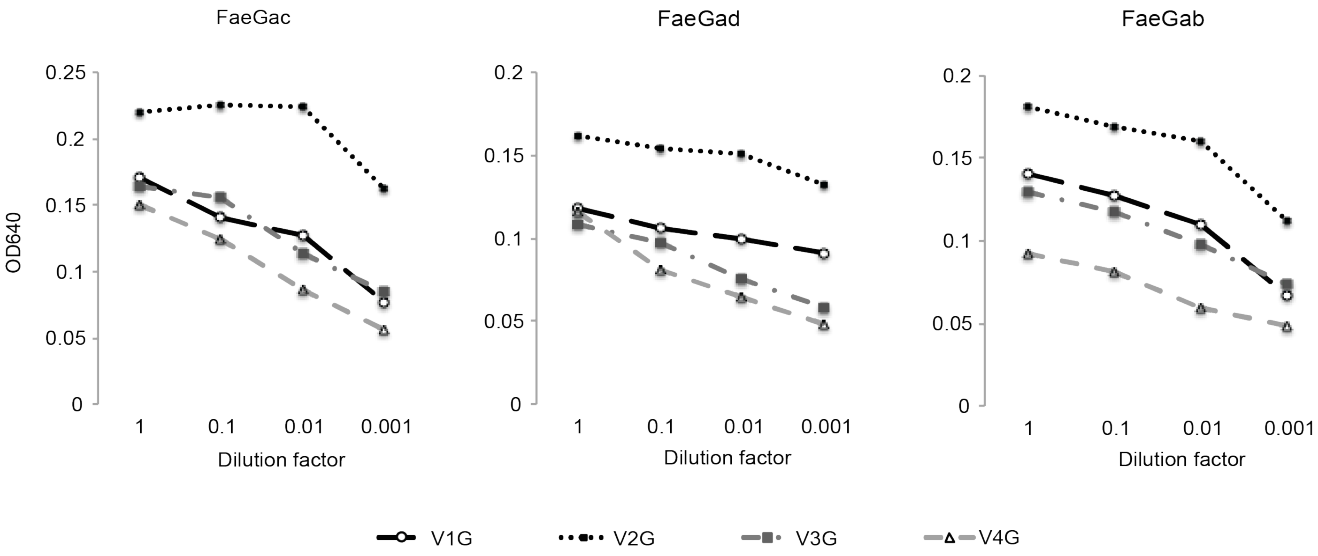


Figure 4.10: All VHH-IgGs recognise the three variants of FaeG in the F4 fimbriae namely- FaeGac (left), FaeGad (middle), and FaeGab (right) in ELISA.

Along with the amino acid sequence, the abundance of a heterologous protein is influenced by the interplay between the host machinery (e.g. chaperon, post translation modification enzymes etc.) and the heterologous protein itself (myristylation in membrane-associated proteins, presence or absence of putative glycosylation sites) (Nuttall *et al.*, 2002; Rybicki, 2010). Antibodies are generally regarded as better accumulators as their proper folding and assembly is facilitated by the ER resident chaperon proteins like the Calreticulin, BiP (binding protein) and ERp57 (Nuttall *et al.*, 2002). Proteins that fail to comply with the endogenous protein production machinery, are often miss-folded, or bear incorrect glycosylation. Such proteins are usually degraded by the ER-associated degradation (ERAD) mechanism (Clerc *et al.*, 2009; Hirsch *et al.*, 2009). This mechanism for degradation of in seed produced single chain antibodies was also suggested by Loss *et al.* on studying the degradation of the anti-HIV single chain antibody 2G12 expressed in Arabidopsis seeds (Loos *et al.*, 2011b). Such excessive degradation or specific proteolytic fragmentation of V3G and V4G (bearing just one amino acid difference), compared to V1G and V2G would explain lower accumulation of fully

functional assembled antibodies. However, the immunoblot analysis with polyclonal anti-Pig IgG antibodies did not reveal excessive proteolitic fragmentation for V3G and V4G as compared to the other two PoLa antibodies V2G and V1G. In fact all the 4 PoLa antibodies showed an identical and specific proteolytic cleavage product of ~37 KDa and 15 KDa, suggesting a proteolytically sensitive amino acid sequence in the common Fc element (Figure 4.9). Seemingly this fragmentation is dose dependent, as the amount of cleaved fragment was proportional with the total antibody amounts; on an average transformants having production level more than ~3% of TSP showed high amount of degradation (Figure 4.9). Dried matured seeds being an end point system, only proteolysis fragments presumably generated by specific proteases can be seen and analysed, however degradation of an unfit protein could be due to the ERAD pathway. Investigation of the VHH-IgG during seed filling (maturation) phase might shed more light into mechanisms, and its involvement in accumulation.

Further, differential glycosylation as another hallmark of posttranslational modifications was seen in the highest expressing lines of V2G and V1G (higher than 10% of TSP) as a faint band migrating underneath the ~49 kDa PoLa antibody band (Figures 4.6 and 4.8). This band appears to be the unglycosylated variant of the highly expressed VHH-IgG, such unglycosylated bands of ScFv-Fc expressed in Arabidopsis seeds have also been reported earlier in high accumulating ER-retained single chain-Fc (ScFv-FC) antibodies (Loos *et al.*, 2011b; Van Droogenbroeck *et al.*, 2007). However unlike in our study, the ratio of glycosylated to unglycosylated bands were almost similar (approximately equimolar doublet), irrespective of the accumulation attained. The results of Van Droogenbroeck et al. (2007) suggested that the doublet of equal ratio visible under reduced condition could be due to glycan attachment to only one of the chain of ScFv-Fc antibody. Our results do not show this equiratio doublet of band. Rather the occurrence of a faint unglycosylated band suggest overwhelming of the glycosylation machinery; where under high production and accumulation pressure a minute fraction of PoLa is left unglycosylated (hence seen only in high expressing lines). We did not study the

glycosylation of these antibodies in detail, glycosylation can be very important for ensuring functionality and stability of an antibody and hence needs to be characterised. In case of parenteral administration plant glycan can lead to undesired glycan specific immune reaction, this issue however is not of an equally impending nature for oral passive immunisation.

Stability of seed produced antibody in crushed seed powder over time

For feed formulation to achieve in-feed passive immunisation, the antibody producing seed would have to be milled. Rupturing the seeds might have an effect on the stability of antibody in seed powder since they would be more liable to external factors. Hence we accessed the fitness of the antibody in crushed seeds over a period of 10 weeks, when stored at ambient room temperature (23°C). Over all, the 4 VHH-IgG antibodies were relatively stable and functional over the period of 10 weeks, as analysed by ELISA (Figure 4.11). The titre of functional antibody in milled seed material only started to drop slightly in case of V3G and V4G in samples incubated for 10 weeks, demonstrating the stability *ex situ* at ambient conditions.

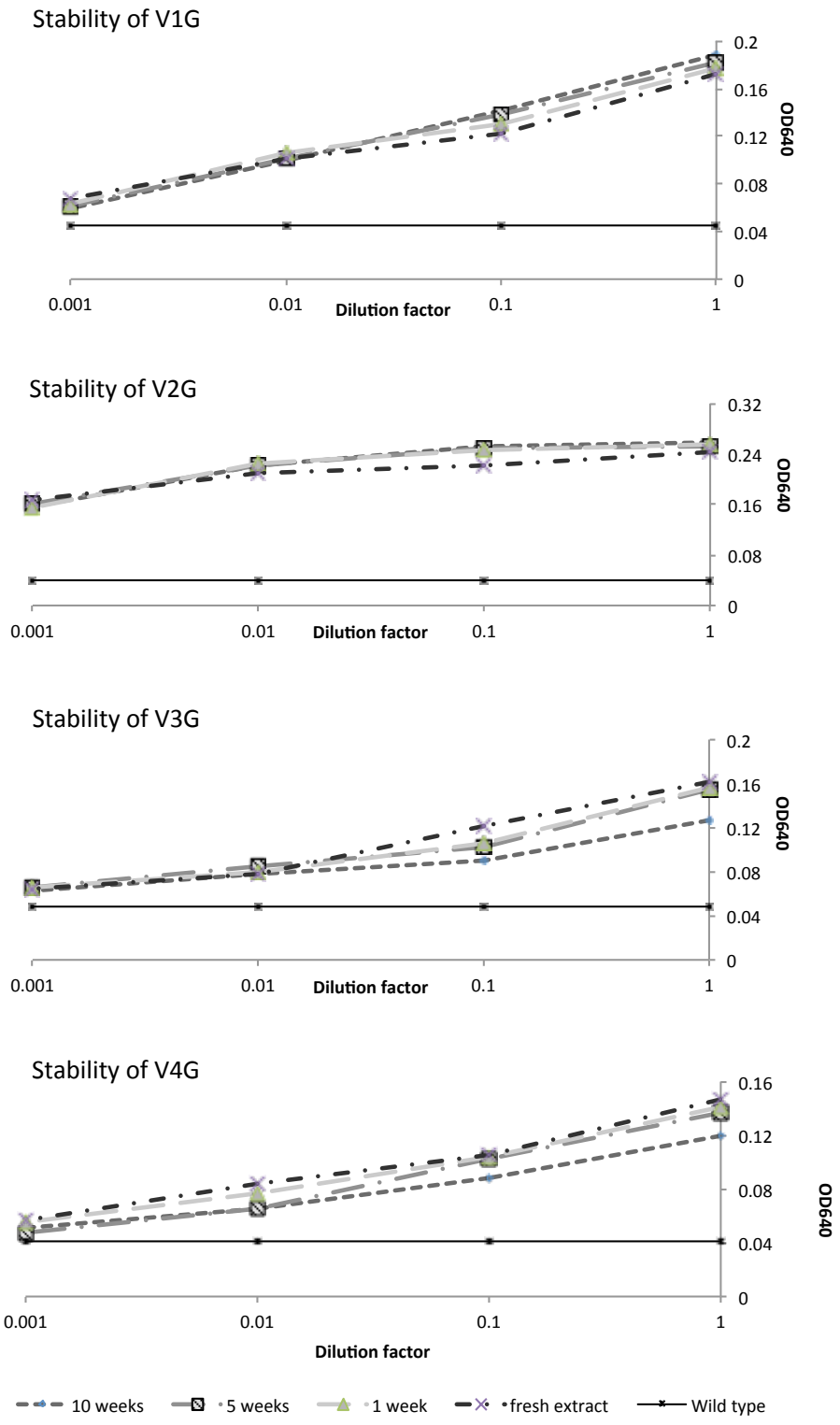


Figure 4.11: All the VHH-Fcs were stable in crushed seed for weeks at room temperature. The titre of the antibody V3G and V4G begins to decline after 10 weeks later.

Seed produced anti-F4 antibodies inhibit bacterial binding to gut villous enterocytes

Protein extracts from seeds producing the anti-F4$^+$ETEC antibodies were tested for their ability to inhibit F4$^+$ETEC (serotype FaeGac) attachment to gut villous enterocytes in an *in vitro* adhesion test (Coddens *et al.*, 2009). Same quantity of total soluble seed protein (identical seed weight) was incubated with the F4$^+$ETEC-FaeGac bacteria [field isolate- GiS26, (Cox and Houvenaghel, 1993)] and then added to the porcine gut villous enterocytes. All seed extracts with the four antibodies inhibited the binding of the bacteria to the gut villous enterocytes (Figure 4.12). However, due to the semi quantitative nature of the functional test, the functional capacity of the 4 antibodies could not be compared (Figure 4.12). The test was repeated with purified antibodies and gave the same result (data not shown), confirming that the antibodies specifically inhibit binding to the gut villi and not an element of the seed extract. Also, for any particular anti-F4$^+$ETEC PoLa antibody higher dilution in dose had consequential lower reduction in inhibition of the bacteria; thus dually confirming the biological relevance.

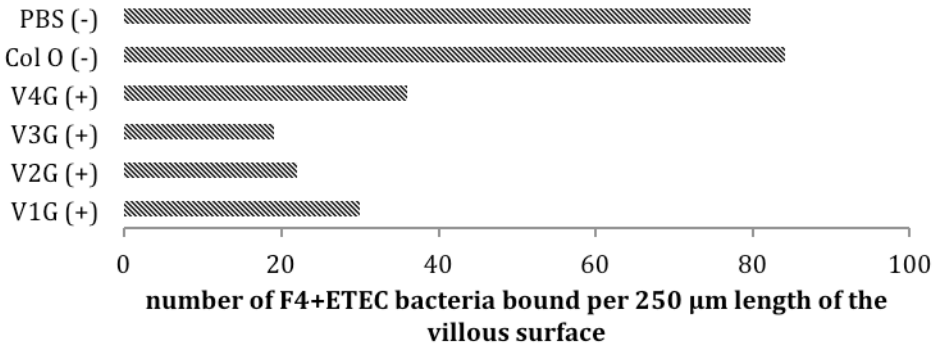


Figure 4.12: All seed made antibodies agglutinate the pathogenic bacteria and inhibits its attachment to gut villous enterocytes. The bars represent the average number of bacteria bound per 250 µm length of the villous surface, as determined by porcine villi binding inhibition test. In this semi-quantitative assay 100 µl of seed extract made from the pool of seeds expressing each of the 4 antibodies V1G (expression in pooled seeds ~ 10% of TSP), V2G (expression in pooled seeds ~15% of TSP), V3G (expression in pooled seeds ~ 2% of TSP) and V4G (expression in pooled seeds ~ 2% of TSP) showed inhibition of Gis26 F4$^+$ETEC bacteria to F4R positive piglet gut villous enterocytes; as compared to same concentration of wild type seed extract (Col O) or 100 µl of phosphate buffer (PBS) as control. All the seed extracts were made by suspending 8 mg of crushed seeds in 200 µl of extraction buffer. The bound bacteria were counted under phase contrast microscope at a magnification of 600 times. Additionally, the presence and absence of bacterial agglutination observed is indicated in parenthesis.

To summarise the *in vitro* efficacy, all the four anti-F4 PoLa antibodies were folded correctly in plant and formed dimeric antibodies (Figure 4.7) which bound to all the three antigenic variants of surface adhesin FaeG (Figure 4.10), agglutinated F4$^+$ETEC bacteria and inhibited the bacterial attachment to the porcine gut villous enterocytes (Figure 4.4 and Figure 4.12), suggesting that all the 4 VHH-Fc bind to epitopes common to the three variants of FaeG, i.e. FaeGac, FaeGab and FaeGad. Contradictory to our results, previous results from Harmsen *et al.* (2005) with monomeric anti-F4ac VHHs suggested that binding to the variable epitope is necessary for inhibiting the attachment of the respective FaeG variant bearing F4$^+$ETEC bacteria. VHHs are predominantly known to interact with non-linear conformational epitopes (Lauwereys *et al.*, 1998; Muyldermans *et al.*, 2001); all the 4 anti-F4$^+$ETEC PoLa antibodies that we produced do not recognise the antigen FaeG under denatured condition in immunoblots (data not shown), implying that the broad spectrum merit of the 4 PoLa in inhibiting the F4$^+$ETEC binding to gut villus is perhaps achieved by binding to certain common epitope that is inaccessible to conventional monoclonal antibodies or the VHH isolated by Harmsen *et al.* (2005) which did bind to FaeG in immunoblots. In all providing that the novel PoLa antibodies can target previously hidden epitopes, and thus further this strategy can be used for designing antibodies against other orphan porcine enteric diseases.

In conclusion, the results of this chapter demonstrate the feasibility for producing 'disease customised' antibodies for specific prophylaxis against F4$^+$ETEC infection designed for oral delivery. The VHH panning strategy against two antigens led to isolation of VHHs that recognised all three serotype of the F4$^+$ETEC bacteria. Further, high level accumulation of dimeric VHH-IgG 'porcinised-lama' antibody was attained. Our insights about accumulation from our results and related discussion will help in planning and designing future functional antibody to ensure high accumulation in seeds. The most important merit of these antibodies is the effective prevention of adhesion of pathogenic F4$^+$ETEC bacteria *in vitro.* Most importantly, the PoLa antibodies expressed in seeds are promising candidates for developing anti-F4$^+$ETEC oral prophylactic therapeutics. Further in Chapter 6 we assess the

efficacy of these antibodies in preventing F4$^+$ETEC infection in a piglet disease model on challenge. We have also transformed two of these anti-F4$^+$ETEC VHH-IgGs in soybean and pea to eventually develop a feed-based prophylactic therapeutic, the prospects of which have been discussed in Chapter 7.

Experimental procedures

Generation of VHHs against adhesion molecule FaeG of F4 bearing ETEC

Immunisation and construction of immune library

Enterotoxigenic *Escherichia coli* (ETEC) strains bearing F4 fimbriae (F4$^+$ETEC) can be serologically classified into three variants, depending upon the variable domain of the adhesion molecule FaeG, as FaeGab, FaeGac and FaeGad (Van den Broeck *et al.*, 2000). Each of these variants has a common epitope 'a' and respective variable epitope 'b, c or d'. Of these three variants, ETEC bacterial bearing FaeGac are predominantly isolated from diseased piglets (~60%) (Chen *et al.*, 2004; Osek, 1999). Hence the FaeGac was chosen as antigen for immunising the lama to generate antigen binding variable domains of heavy chain only antibody (VHH). Purified recombinant FaeGac molecule was used to immunise a lama. Six weeks later the lymphocytic cDNA library was made. Primers specific to the variable domain of the heavy chain only antibody (VHH) were used to amplify the VHHs coding sequences. These amplicons bearing VHH coding sequences were then cloned into the pMES4 vector and transformed into TG1 cells and thus a VHH library was generated. The different steps are elaborated in detail below. For detailed step wise protocol see Hassanzadeh-Ghassabeh *et al.*, 2011.

Phage display

A 100 µl aliquot of the VHH library was inoculated in 100 ml of 2 x TY medium (bacto-tryptone 16 g, bacto-yeast extract 10 g, NaCl 5 g per litre; at pH 7 adjusted with NaOH; sterilised by autoclaving at 1 bar pressure for 20 minutes) supplemented with 100 µg/ml ampicillin and 1% glucose. When the OD_{600} was approximately 0.1, the inoculated medium was incubated at 37°C, on a shaker with 250 rotations per minute for 2-3 hours until the OD reached 0.5-0.6, indicative of the exponential growth phase of bacterial cells expressing F-pilus. At this point 10^{12} plaque forming units of the M13K07 helper phages were added to the cell culture and maintained stationary at room temperature for 30 minutes; this enables the phage particles to infect the bacterial cells via the F pilus. Post incubation, the cell

culture was centrifuged at 2000 rpm (eppendorf, 5810 R centrifuge, Germany), the medium was removed and the pellet was then resuspended into 300 ml of the 2 x TY medium now supplemented with 100 µg/ml ampicillin and 70 µg/ml kanamycin to select for phage infected cells, which in turn produce phage particles displaying VHH on their surface. This culture was incubated overnight at 37°C on a shaker with 250 rotations per minute; subsequently the phage particles displaying VHH were harvested and used for panning.

Harvesting phage particles

The 300 ml bacterial cell culture infected with M13K07 phage particles grown overnight was centrifuged at 8000 rpm at 4°C (Beckman coulter, Avanti J-20 centrifuge, USA) to pellet the bacterial cells from the supernatant with the phage particles. The phage particles from the supernatant were precipitated by adding 1 part sterilized 20% polyethylene glycol (PEG6000) with 2.5 M NaCl to every 4 part of supernatant (e.g. 10 ml PEG-NaCl to 40 ml supernatant) and mixed by inverting and then maintained on ice for 30 minutes. This mixture was then centrifuged at 4000 rpm (JA rotor) for 30 minutes at 4°C to obtain pellet of phage particles, the supernatant was removed and the pellet was air-dried. This pellet was re-suspended in limited volume of phosphate buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in one litre distilled water, pH 7.4, sterilised in autoclave) (approximately less than 1 ml) and centrifuged at 14000 rpm for 2 minutes (Eppendorf desktop centrifuge) to clear any bacterial debris and phage aggregates. The phage particles (supernatant) were then transferred to fresh microcentrifuge tube and the concentration of the phage particles was measured by determining the OD at 260 nm, where OD of 1 accounts for 3 x 10^{10} phage particles/ml.

Antigen panning

Using phage display technology (Smith, 1985; Winter *et al.*, 1994), the phage particles displaying the VHH on their surface were panned against the antigen FaeGac and FaeGad for detecting VHHs specific for the predominant ‘c’ epitope as

well as the common 'a' epitope of the three FaeG variants. Wells of microtiter plates were coated with the recombinant adhesion proteins FaeGac and the FaeGad, at a concentration of 100 ng per well in 100 µl carbonate buffer. These plates were incubated at 4°C overnight, and the next day the wells were washed 5 times with PBS containing 0.05% Tween20. After washing, the wells were blocked with 2% skimmed milk solution in PBS, for 2 hours at room temperature. Apart from the wells coated with antigen, additional uncoated wells were blocked as negative control to determine the unspecific bound phage particles. After blocking, the wells were washed again 5 times with PBS containing 0.05% Tween20. Then the 10^{11} phage particles displaying the VHH on their surface were added per well (both coated and uncoated), and incubated for an hour at room temperature. Then, the unbound phage particles were removed (discarded in bleach) and the wells were washed 15 times with PBS containing 0.05% Tween20, to remove non-specifically bound phage particles. The bound phage particles were then eluted out by adding 100 µl of 1% (130 mM) triethylamine (pH 10) (Sigma-Aldrich) in the wells, incubating for 20 seconds and the 100 µl elution was immediately transferred into vials containing 100 µl 1 M Tris buffer pH 7.4 to neutralise the eluted phage particles, this was called 'T-elution'. The wells were also immediately neutralised with 1 M Tris pH 7.4 and then washed 5 times with PBS containing 0.05% Tween20. To these wells, 200 µl of TG1 cells (grown overnight in TY medium) in exponential growth phase (OD600- 0.5 to 0.6), were added and incubated at 37°C for 30 minutes. During this incubation any phage particles still bound in the well infects the TG1 cells, which ensures further elution of any remaining phage particles; this elution was called 'C-elution'. From both elusions, 10 µl was utilised for determination of enrichment factor.

The remaining fraction of both the C-elution and T-elution were used to further amplify the selected phages and thus enriched for specific binders in every subsequent panning rounds. The T-elution was added to 2 ml TG1 cells and the C elution to 1 ml 2x TY medium and incubated for 30 minutes at 37°C. Subsequently, 8 ml of 2xTY medium supplemented with 100 µg/ml ampicillin and 20% glucose was

added to both the cell cultures and incubated for another 30 minutes at 37°C. The re-amplification of the phages was done similarly as described in section phage display above and is elaborated in detail in next section. The eluted phage particles were used in subsequent round of panning. The panning was repeated until specific phages were enriched. To determine the enrichment factor an aliquot was kept from each round and evaluated by ELISA and colony count.

Re-amplification of eluted phage particles for successive round of panning

The eluted phage particles (~190 µl) were added to 2 ml of TG1 cells (OD600= 0.5-0.6) in a 15 ml Falcon tube, and incubated at 37°C for 30 minutes; to these infected TG1 cells 8 ml of 2xTY medium (with 100 µg ampicillin and 1% glucose) was added and incubated at 37°C on a shaker for 30 minutes. Then 10^7 M13K07 helper phages were added to the cells and incubated at room temperature for 20 minutes without shaking. Subsequently, the cells were pelleted by centrifuging at 2000 rpm in a swing-out rotor to remove glucose from the medium (glucose removal prevents leakage from lacZ promoter, upstream on VHH gene). The cell pellet was then resuspended in 2xTY medium with ampicillin (100 µg/ml) and kanamycin (70 µg/ml) in 250 ml volume and incubated on a shaker with 250 rpm at 37°C during the night; this overnight culture was then used for panning.

Determination of enrichment factor by colony count

To determine the enrichment factor after each round of panning, 10 µl of the eluted phage particles (t-elution) from the wells coated with antigen and uncoated wells were taken and diluted 10 fold serially with PBS in a microtiter plate. In another round bottom microtiter plate TG1 cells were seeded in 90 µl of TY medium (OD600 was around 0.5-0.6). To these cells (90 µl) 10 µl of each of the phage dilution was added and incubated at 37°C for 1 hour to infect the TG1 cells. After incubation, 10 µl of these infected cells corresponding to each dilution were plated on LB-agar (Lysogeny broth media- 0.5 g/L yeast extract, 1% tryptone, 1% NaCl and 1.5% agar) plates with 100 µg/ml ampicillin and 1% glucose. As negative control 10 µl of the uninfected TG1 cells were also plated on LB-agar culture plates. On the same

principle, the C-elution was also 10 fold serially diluted and then each dilution was plated directly on a similar LB-agar culture plates (with 100 µg/ml ampicillin and 1% glucose). These culture plates were incubated overnight at 37°C, and the next day the enrichment factor was determined by counting the colonies. Every successive round of panning let to enrichment of the specific VHHs displaying phages, i.e. the number of colonies representing the specifically bound phages. Thus the enrichment of phages expressing specific VHHs on their surface was determined, for each round of panning.

Determination of enrichment factor by phage ELISA

After each round of panning a phage ELISA was performed to determine the enrichment of the specifically binding phages in addition to colony count. Similar to the antigen panning set up as described above, wells of the microtiter plates were coated with FaeGac antigen or with FaeGad (100 µl per well, concentration of 1 µg/ml in carbonate buffer (0.1 M $NaHCO_3$, pH 8.2) by incubating overnight at 4°C. The wells were washed 5 times with PBS containing 0.05% Tween20. The coated wells along with additional negative control wells were blocked with 2% skimmed milk in PBS, for 2 hours at room temperature. These wells were subsequently washed 5 times with PBS containing 0.05% Tween20, and 10^{10} phages were added from each rounds of panning, in the coated wells as well as in uncoated wells, and incubated for 1 hour at room temperature. Subsequently, the wells were washed and 100 µl of 1/1000 diluted anti-M13 antibody conjugated to horseradish peroxidise (Amersham) was added and incubated for another hour at room temperature. Wells were then washed and 100 µl of substrate 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) was added into each of the wells. The enzyme-substrate reaction leads to development of yellow soluble product, the intensity of which was measured at 405 nm; the absorbance corresponds to the amount of bound phage particles.

Selection of independent FaeG binding VHH

Expression of VHH

From the LB-agar plates used for determining enrichment factor, individual colonies were picked from each dilution, replicated on a fresh LB-agar plate (for further reference) and inoculated in wells of a 24 well titer-plate filled with 1.5 ml LB medium supplemented with 100 µg/ml ampicillin. These inoculated 24 well titer-plates were incubated for 3 hours at 37°C (until a clear pellet was formed at the bottom of the wells). Then the VHH expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG, Fermentas) to a final concentration of 1 mM; these induced cultures were incubated wile shaking at 28°C overnight. Within the expression cassette, the periplasmic signal peptide PelB was attached to the VHH, leading to the accumulation of VHH in the periplasmic space of the bacterial cell. The next day the 24 well plates were centrifuged for 15 minutes at 2800 rpm (A-4-81 MTP/flex rotor, Eppendorf, 5810 R centrifuge, Germany), at 4°C. The medium was removed, and the pellet were resuspended in 150 µl TES buffer (0.2 M Tris, 0.5 mM EDTA, 0.5 M sucrose- pH8) and incubated on a shaker for 30 minutes at 4°C. After which, 250 µl of TES/4 buffer (0.25% TES) was added per well and incubated again while shaking for 30 minutes at 4°C. The 24 well plates were then centrifuged for 15 minutes at 2800 rpm (A-4-81 MTP/flex rotor, Eppendorf, 5810 R centrifuge, Germany) at 4°C. This change in concentration of sucrose (TES) leads to an osmotic shock and the periplasmic extract bearing the VHH is released. This periplasmic extract was then used in the ELISA plates coated with antigen as described below, for confirmation of its specific binding to the target antigen.

VHH-antigen ELISA

The antigenic variants of FaeGac and FaeGad were coated in 2 individual 96 well multi-titer plates. Each of the antigenic variant was diluted to 1 µg/ml concentration in 1 M carbonate buffer (pH 8.2); 100 µl of this solution was added per well and incubated at 4°C overnight. The following day, the coating solution was decanted and the plates were washed 5 times with PBS containing 0.05% Tween20 (washing

solution). The wells were then blocked for 2 hours at room temperature with 200 µl of 2% skimmed milk in PBS. After blocking, the wells were washed 5 times with the washing solution and then 100 µl of the periplasmic extract (bearing VHH) was added into the wells and incubated at room temperature for 1 hour. The wells were washed 5 times with washing solution, and then 100 µl of primary antibody diluted 1/1000 in 2% skimmed milk was added. In the wells coated with FaeGac anti-Haemagglutinin-tag monoclonal (mouse) antibody (BABCO) was used as the primary antibody. While in the wells coated with FaeGad, the primary antibody used was anti-his-tag monoclonal (mouse) antibody. The plates were incubated at room temperature for 1 hour with the primary antibodies, and then washed 5 times with washing solution. Subsequently, 100 µl of a 1/1000 dilution of anti-mouse monoclonal antibody conjugated to alkaline phosphatase (Sigma) was added to both the plates and incubated for 1 hour. Finally, the wells were washed again 5 times with washing solution and the ELISA was developed by adding 100 µl of the substrate – 2 M disodium-p-nitrophenyl phosphate (dNPP, Sigma) in each well. After 10 to 20 minutes, the OD405 of the wells was measured. The wells with signal at least twice the signal of the control wells were accepted as being FaeG-specific VHH. The colonies producing these specific VHHs were further screened by PCR.

Confirmation by PCR

The colonies corresponding to the VHH that gave positive signal in the ELISA screen were further tested by amplifying the VHH-gene, to identify and select distinct VHHs. The positive colonies from the reference LB medium plate were dissolved in 50 µl sterile water and 5 µl from this dilution was added to the PCR mixture [PCR mixture: 11,75 µl sterile filtered water, 5 µl ex TaKaRa 10x buffer (TAKARA Bio Inc, Japan), 1 µl 10 mM dNTP (TAKARA Bio Inc., Japan), 1 µl 10 µM GIII primer, 1 µl 10 µM MP57 primer and 0.25 µl TAKARA ExTaq enzyme (TAKARA Bio Inc., Japan)]. PCR was performed on a GeneAmp PCR system 9700 (Applied Biosystems, USA) with the program as: initial denaturation at 94°C for 2 minutes, followed by 30 cycles of (1^{st}) denaturation step at 94°C for 30 sec, (2^{nd}) annealing step at 55°C for 30 sec, (3^{rd}) elongation step at 72°C for 45 sec, and then a final elongation step for 5

minutes at 72°C. The amplified fragments were separated and analyzed by agarose electrophoresis. For this, 5µl PCR product, together with 2 µl DNA loading buffer were loaded on a 1% agarose gel in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) and separated at 110 V for 25 minutes. As marker, 5 µl λ PstI or smartladder was used. The gel was stained with ethidium bromide and visualization under UV light (For more details, and tips see Hassanzadeh-Ghassabeh *et al.*, 2011).

MP57 primer: 5'-TTATGCTTCCGGCTCGTATG-3';

GIII primer: 5'-CCACAGACAGCCCTCATAG-3'

Restriction fragment length polymorphism (RFLP)

The positive colonies, which gave a band of 680 bp on agarose gel electrophoresis, were further analysed by RFLP to eliminate similar redundant VHHs. For this, 6 µl of PCR product was digested with 3 units of restriction enzyme for 2 hours at 37°C. The digested PCR fragments were separated on 2% agarose gel as described above. The patterns obtained by HinfI digest (Fermentas) and RsaI digest (Fermentas) were analysed. Similar RFLP patterns were clubbed into one category; several such categories were defined, narrowing down the number of prospective distinct VHHs.

Confirmation by control ELISA

The best positive colonies chosen for sequencing were again checked via ELISA. These selected colonies were plated out again to obtain individual colonies. VHHs were produced from this starting material and then used in the ELISA as described above. On dual confirmation the individual colonies were used to start cultures for glycerol stock. Thus 4 VHHs were selected. These VHHs were named V1, V2, V3 and V4.

Agglutination assay

Covalent conjugation of VHH to the magnetic beads

To evaluate if the VHH were functional in agglutinating the bacteria, the classical agglutination test was performed where the VHH were covalently linked to magnetic beads (Dynabeads M-270 carboxylic acid) in order to make them

multivalent. Firstly the VHHs (previously in PBS) were dialysed with 50 mM MES pH 5.0, using the Vivaspin500 columns (molecular weight cut off ~5 kDa) and concentrated to ~1 mg/ml concentration. This buffer exchanged VHH were then chemically conjugated to the magnetic beads in accordance to the manufacture's instruction. Briefly, the beads were washed twice with 0.01 M NaOH, and thrice with deionised water, using a magnet the supernatant could be easily decanted between each washing step while retaining the magnetic beads in the microcentrifuge tube. For every VHH, 100 µl of washed bead suspension (~3 mg) was used. To this 100 µl bead suspension 200 µl of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added and incubated for 30 minutes on rotating wheel, to activate the ligand. The beads were then washed firstly with equal volume of ice-cold water followed by ice-cold 50 mM MES pH 5.0. The wash solution was decanted, and the activated beads were incubated with 60 µg of respective VHH to be conjugated in 100 µl of 50 mM MES pH 5.0, at room temperature for 30 minutes on rotating wheel. The unbound VHHs were then washed away and the beads were blocked with 50 mM Tris pH 7.4 for 15 minutes at room temperature. Finally the VHH coated beads were washed 3 times with PBS containing 1% Tween 20 (v/v), and stored in this final wash buffer at 4°C.

Growing of F4+ETEC bacterial culture for agglutination assay

The $F4^{+}$ETEC strains (C585-80 expressing F4ad fimbriae, C95-72 expressing F4ac, C1023-78 expressing F4ab) and the negative control strain K514-PIH120 were streaked on LB agar plates and from this a single colony was inoculated in 20 ml of liquid LB medium and the flask was maintained stationary at 37°C for 2 days. The bacterial cells were pelleted and washed twice and then resuspended with PBS to an OD600 of 16 to 21.

Agglutination on glass slides

On a clean microscope glass slide 10 µl each of the bacterial culture and the VHH coated magnetic beads were added at the same spot, and the two droplets were mixed together. The presence or absence of agglutination (Figure 4.4) was observed

and noted after incubation of one minute during which the slide was gently rocked.

Fusion of the VHH to Pig Fc (Porcinised-camelid chimeric antibodies)

Six classes of porcine IgG have been identified. Of these the porcine IgG3 has the longest hinge with three cysteines residues and is predicted resistant to peptic degradation, hence the IgG3 Fc was chosen for fusion with VHH aiming at designing a robust molecule. The coding sequence of the porcine IgG3 gene (accession no.:EU372658) was retrieved and the hinge, CH2 and CH3 coding sequence was codon optimised. The codon usage within the native sequence of porcine IgG3 Fc was compared to that of Arabidopsis seed storage proteins (2s1: At4G 27140, 2s2: At4g 27150, 2s3: At4g27160,2s4: At4g 27170, Cru: At4g28520, Cra1: At5g44120, CRB: At1g03880 and CRU2:At1g03890) using Cusp (European Bioinformatics Institute, UK). The codon usage of the heterologous protein was then manually optimised to seed storage proteins with an intention to achieve high accumulation levels.

In silico the DNA sequence of VHH V1 was fused with the codon optimised sequence of IgG3 Fc, upstream of this construction the sequence of EcoRI restriction site, Kozak sequence (CCACC) and the 2S2 seed storage signal peptide sequence were added in the same order, while downstream of the VHH-Fc fusion a KDEL endoplasmic retention signal, stop codon and BamHI restriction site were introduced. This entire stretch of DNA sequence was chemically synthesised flanked with the Gateway attB1 and attB2 sites and cloned within the multiple cloning sites of the pUC57 vector (Genscript). In a Gateway BP reaction, this pUC57 vector bearing the construct within the attB sites was recombined with the Gateway Donor plasmid pDon221 (bearing kanamycin resistance) according to manufacturers instruction, which results in an entry clone, which was named pEV1G. Plasmid DNA from pEV1G was then transformed into chemically competent *E. coli* (strain DH5α), and plated on LB medium with kanamycin (50 µg/ml). Positive colonies bearing the expected V1-IgG3 fusion were screened by studying the restriction digestion pattern and confirmed via sequencing.

For cloning of other three anti-F4+ETEC VHHs– V2, V3 and V4 in fusion with IgG3 Fc the DNA sequence for restriction site for EcoRI + Kozak sequence + 2S2 seed storage signal peptide + each of the three VHHs was chemically synthesised. Then Using the EcoRI and the BstEII (located in framework4 of all VHHs) restriction sites, the VHHs V2, V3 and V4 with the 2S2 signal peptide were swapped with the V1 from entry clone pEV1G. Thus, Entry clones (E) pEV2G, pEV3G and pEV4G were made.

In seed expression of anti-F4+ETEC antibodies

Gateway recombination into pPhasGW vector and *Agrobacterium* mediated transformation

All the above describes entry clones were recombined via an Gateway LR reaction into the destination vector pPhasGW (Morandini *et al.*, 2011) following the Gateway instruction manual. The pPhasGW destination vector bears a seed specific β-Phaseolin promoter and 3' arcelin terminator bearing regulatory sequences, which enables high seed specific expression of heterologous proteins (Morandini *et al.*, 2011). The T-DNA also bears the *nptII* gene, which confers for kanamycin resistance in plants and thus facilitates screening of transformed plants. On LR recombination reaction (Gateway), expression clones were obtained; these clones were checked via restriction digestion and further confirmed via sequencing. The 4 expression clones (X) bearing the VHH-IgG fusion were named: pXV1G, pXV2G, pXV3G and pXV4G.

All the expression plasmids bearing correct sequence were then transformed into the *Agrobacterium* strain C58C1Rif^{R} with helper plasmid pMP90 (Koncz and Schell, 1986) via electroporation of 50 ng plasmid DNA in 40 µl of competent cells within a 1 mm electroporation cuvette (Biorad) at 2.2 kV pulse. The *Agrobacterium* colonies were then selected on YEB (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM solution MgSO4) plates (1.5% agar to YEB medium) with antibiotics streptomycin (20 µg/ml), spectinomycin (100 µg/ml) and rifampicin (25 µg/ml). These colonies were further checked via restriction digestion and sequencing of plasmid DNA. Single colonies with the correct plasmids were used to start cultures for glycerol stock and *Agrobacterium* mediated floral dip

transformation.

Agrobacterium mediated floral dip transformation of Arabidopsis

The *Agrobacterium* strain bearing plasmid with correct sequence was used for floral dip transformation (Clough and Bent, 1998). A single colony was picked from the respective construct to be transformed and inoculated into 1 ml of liquid LB medium with rifampicin, gentamycin, streptomycin (each 20 µg/ml) and spectinomycin (100 µg/ml) as selection antibiotics. This culture was allowed to grow for 8 hours at 28°C on a rotary shaker with 280 rpm, after which 10 ml of LB was added to the culture and incubated overnight again at 28°C and 280 rpm. The optical density of the *Agrobacterium* culture was measured at wavelength of 600 nm. This OD600 should ideally be 1.7. This 10 ml culture was then made up to final volume of 50 ml with dipping solution (10% sucrose and 0.05% Silwet solution in water). The flowers of Arabidopsis plant were dipped in respective *Agrobacterium* solutions for 10 seconds, after which the plants were covered with a cellophane wrap to maintain humidity and kept in growth room with 16 hours light and 8 hours dark regimen. Five plants were dipped for each of the 4 VHH-IgG. After 24 hours the cellophane wrap was removed and the plants were allowed to grow until the ripe siliques become dry (~6weeks). The T1 seeds were then harvested.

Selection of T1 transformant plants

The T1 seeds were harvested from the floral dipped plants, and approximately 1000 (~25 mg) seeds per plant were sterilized via vapour phase method. Seeds were weighed in a micro centrifuge tubes, and the tubes were placed with their lids open in a bell jar of 20 litters volume kept in the fume hood. Before closing the lid of the bell jar a beaker with 100 ml bleach (12% sodium hypochlorite) was kept in the jar, the lid of the jar was then partially closed leaving as little room for a pipette to pass. Through this narrow opening 3 ml of concentrated HCl was added and the lid was immediately closed tight. The seeds were left in the chlorine fumes for 14-15 hours, after which the micro centrifuge tubes were closed again and brought to sterile laminar airflow where the lids were open to let out the chlorine fumes for at least an

hour. Sterilised seeds were sown on large Petri plates (150 mm x 25 mm) with sterile Murashige and Skoog medium [4.308 g/L Murashige and Skoog salts, (Gibco BRL, Gaithersburg, MD) with 0.5 g/L of MES (Duchefa), 10 g/L of sucrose), pH 5.7]. Prior to sterilisation 0.9% plant agar was added and the medium was sterilised at 121°C for 20 minutes at 1 bar pressure. After the medium cooled to about 60°C, the vitamins and antibiotics [B1-Vitamins (1 ml/l), nystatin (50 mg/l), vancomycin (750 mg/l) and 50 mg/L kanamycin (Duchefa)] were added in a sterile laminar air flow, mixed by swirling and then poured in the Petri plates to a height of ~1cm. These plates were then sealed with porous adhesive tape (Milipore), which facilitates exchange of gases. The plates were then maintained in growth chambers with 21°C under fluorescent (cool white) light 80 $\mu E/m^2/s$ for 16 hours light and 8 hours dark photoperiod. The temperature of the shelves was maintained at 19°C to prevent moisture condensation on the lids of the plates. By 3-4 weeks primary transformant seedlings were transferred to soil.

Characterisation of in-seed made anti-F4$^+$ETEC antibodies

<u>High through-put protein extraction from T2 seeds</u>

The protein extraction was performed in strips of 8 attached microtubes; this format is compatible with (8 X 12) micro-racks, allowing simultaneous extraction from 96 plant lines. Five-milligram seeds were weighed per plant in each of the individually labelled microtube. Intermittently as needed the tubes and the scale was sprayed with an ion gun, to avoid any cross contamination due to static developed on the seeds. One steel ball of 4 mm was added into each microtube, the tubes were closed and snap chilled in liquid nitrogen. The frozen seeds were crushed in a mill (Mixer mill MM400-Retsch) for 20 seconds at 20 Hz frequency of oscillation. Immediately 600 µl of cold extraction buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.1% Tween 20) was added to the crushed seeds and vortexed generously to suspend all the seed powder and dissolve the soluble fraction. The 96 tubes micro-rack was centrifuged at 4°C for 10 minutes at 3310g (Eppendorf- swing out rotor). The rack was maintained on ice after centrifugation, 300 µl of protein extract was taken (middle clear phase) without disturbing the

pellet at the bottom and the oil layer on top. To this 300 µl protein extract 20% glycerol was added before storing at -20°C.

Measurement of total soluble protein in seed extracts

The total soluble protein (TSP) concentration of seed extract was measured by Bio-Rad DC™ protein assay kit. This is a detergent compatible, colorimetric assay kit based on Lowry assay, which enables protein measurement in 15 minutes. The TSP extracts from seed were diluted 2.5, 5, 10 and 20 times in Mili-Q grade water and 5 µl of these dilutions were added in 96 well flat bottom microtiter plates (NUNC-269620). Also in the same plate, 5 µl bovine serum albumin (BSA) with concentration of 0.2 mg/ml, 0.4 mg/ml, 0.8 mg/ml and 1.6 mg/ml were added as standard and water as blank, negative control. To this loaded plate, 25 µl of reagent A and 200 µl of reagent B was added per well (according to the manufactures instruction) and the reaction mix was incubated at room temperature in dark for 15 minutes. Like Lowry assay the first step of the reaction involves copper-protein interaction in alkaline medium, followed by subsequent reduction of the Folin's reagent by this copper treated protein, which leads to development of blue colour. This colouration was measured using a plate reader (VERSAmax, Molecular Devices, USA) at wavelength of 750 nm and using the versa max software the protein concentration of the seed extract was calculated from the slope of the BSA standard dilutions. For subsequent work, all the protein extracts were diluted to 1 mg/ml concentration.

Determination of expression levels of functional molecules in seeds via FaeGac ELISA

The multisorb ELISA plates (Nunc 439454) were coated overnight with 1 µg/ml concentration of purified recombinant FaeGac in $NaHCO_3$ buffer pH 8.2 in a volume of 100 µl per well at 4°C. Subsequent morning the coating solution was decanted and the wells were washed 5 times by flushing and decanting the plate with 300 µl of 0.1% Tween20 in PBS and finally knocking the plate on absorbent paper to remove residual droplets. The wells were then blocked with 150 µl of 2% skimmed

milk in PBS and incubated at room temperature (25°C) for 90 minutes. Plates were washed again and 100 µl serial dilutions of plant extracts (typically 1/100, 1/200, 1/400, 1/800) made in 2% skimmed milk in PBS were added to the well, incubated for 90 minutes at room temperature and the wells were washed again as described. To detect the bound, seed produced monomeric antibodies anti-Fc specific primary antibody was used. For detecting the plant produced VHH-IgG antibodies 100 µl of polyclonal anti-porcine IgG produced in rabbit (Sigma A5670) conjugated to horseradish peroxidise (HRP) diluted 1/40,000 in 2% skimmed milk was used. After addition of the primary conjugated antibody, the plate was incubated for 90 minutes at room temperature. Finally, after washing, 100 µl of substrate TMB (3,3',5,5' tetramethylbenzidine, Sigma) was added per well, the plates were kept in the dark for 30 minutes and the intensity of the blue coloration developed was measured at 640 nm (VERSAmax, Molecular Devices, USA). The signal intensity being the read out of the functional antibody enabled comparison of relative accumulation in different primary transformants. These plant extracts were then analysed using SDS-PAGE and immunoblot techniques, and plant lines bearing single locus were determined.

NB: The same ELISA set up and primary conjugated antibody was used for determination of stability of antibody in crushed seeds over time (Figure 4.11) and for evaluation of the 4 VHH-IgG antibodies binding interaction with the three FaeG variants (Figure 4.10). In this latter experiment the ELISA plates were coated with 1 µg/ml concentration of purified, recombinant produced, respective FaeG variant viz. FaeGac, FaeGab and FaeGad in $NaHCO_3$ buffer, as described. The purified FaeG variants were provided courtesy of Prof. Henri De Greve.

SDS-PAGE and immunoblot analysis

The characterisation and quantitation of the seed produced antibodies via sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) as well as immunoblotting, was done as previously described (De Buck *et al.*, 2011). In short, the seed proteins were electrophoretically separated on a conventional 12% polyacrylamide gel (for 10 ml 12% SDS-PAGE: 4 ml of 30% 29:1 Acrylamide/bis,

3.75 ml 1 M Tris-HCl pH 8.7, 100 µl 10% SDS, 100 µl 10% APS, 10 µl TEMED, 2.05 ml H_2O) in Tris-Glycine-SDS buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3) at 180 volts for an hour. On electrophoreses, the gels were stained with Coomassie R-250 solution (0.3 mM Coomassie Blue R-250, 40% v/v methanol, 7% v/v acetic acid) overnight and then destained with destaining solution (5% v/v methanol, 7%v/v acetic acid) until the desired contrast of band intensity was achieved. The gels were then documented using the Chemidoc™ system (Biorad) and the concentration of the proteins was determined from the band intensity in comparison to purified antibody standard with Image lab software.

For immunoblotting the SDS-PAGE separated proteins were blotted onto activated PVDF membranes, in a Mini-Protean II™ wet blot system (Biorad) with blotting buffer (14.14 g glycine, 3.024 g Tris, 15% methanol) at 50 V of potential difference for an hour. The blotted membranes were blocked with 2% skimmed milk in PBS at 4°C overnight. Afterwards, the membrane was washed 3 times with 0.1% Tween20 in PBS, incubated with primary antibodies diluted in 2% milk for 90 minutes and washed again 3 times. In case the primary antibody was not conjugated to the enzyme then a secondary antibody was used diluted in 2% skimmed milk and incubated for 30 minutes. To visualise the immobilised target seed produced antibody the chemiluminescent substrate (Western Lightning™ Chemiluminescence Reagent Plus, Perkin Elmer) was used according to manufacturers instruction. This substrate reacts with the HRP to produce a luminescent product indicative of the target protein, which was documented either by using photographic films in a dark room or by using the Chemidoc™ system (Biorad).

For detection of the seed produced VHH-IgG the anti-porcine IgG produced in rabbit (Sigma A5670) diluted 1/4000 in 2% skimmed milk was used.

Transient expression in *Nicotiana benthamiana* leaves

The reference purified V2G^t antibody, used for quantification (Figure 4.7, 4.8 and 4.9) was produced transiently in *Nicotiana Benthamiana* leaves using the pEAQ (Sainsbury *et al.*, 2009) vectors. Briefly, entry clones of the EV2G^t was made as

described above using the pDON207 (gentamycin selection); this entry clones was recombined with the pEAQ-HT-Des1 vector (Sainsbury *et al.*, 2009) according to Gateway manual, the resultant expression plasmids named- TXV2G was transformed into the Agrobacterium strain C58C1 Rif and infiltrated into *N. benthamiana* leaves, the details of this protocol and protein extraction method has been described in De Buck *et al.*, 2011. The V2G antibody was purified from the soluble leaf proteins using protein A affinity chromatography as described above.

Affinity purification of seed produced antibodies

For purification of Arabidopsis seed made antibodies, commercially available affinity based resins were used. For purification of the VHH-IgG antibodies, Protein A sepharose columns (HiTrap rProtein A FF, GE healthcare) were used in AKTA™ liquid chromatography system. All the chromatographic steps were performed at 4°C. First, the column was equilibrated with about 10 column volumes of binding buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.1% Tween 20). Then, the seed extract made in protein extraction buffer (pH 8), was filtered through 0.45 µm filter and loaded on to the column at a flow rate of 1 ml/min without exceeding pressure of 0.2 Mpa. After passing the protein solution, the column was equilibrated again with 10 column volumes of binding buffer and washed twice; first with 100 mM Tris-HCl pH 8 followed by 10 mM of Tris-HCl pH 8 (10 column volumes each). The bound antibodies were eluted using 0.1 M of Glycine buffer at pH 3. Fractions of 450 µl were collected into microcentrifuge tubes pre-dispensed with 50 µl of 1 M Tris pH 8 to immediately neutralise the acidic pH of the eluted fraction. Fractions bearing the purified antibodies were identified by measuring the OD_{280} and such fractions were then pooled. The column was immediately neutralised by washing with 100 mM Tris pH 8 (10 column volumes) and then preserved in 20% ethanol.

Selection and characterisation of plant lines expressing high amount of antibodies

Determination of single locus

For the monomeric VHH-IgG producing plants, the highest expressing lines were selected and 64 surface sterilised seeds from each of these lines were sowed on MS plates with kanamycin. The plates with seeds were sealed with porous tape (Millipore), kept in dark at 4°C for 48 hours for stratification and transferred to growth room at 21°C with 16 hours light and 8 hour dark photoperiod. After 2-3 weeks the resistant plants could be well distinguished from the sensitive ones. These numbers were compared via chi square test to determine the lines with significant 3:1, resistant : sensitive segregation pattern, which is indicative of single locus insertion (De Neve *et al.*, 1997).

Determination of homozygous plants

On determining the lines with single locus insertion all the seedlings from such single locus plants were transferred to the soil in the green house (16 hours light and 8 hour dark photoperiod). After 4-6 weeks the T3 seeds were harvested from the plants and approximately 1000 seeds (~25 mg) were vapour sterilised and sowed on the K1 media. Monomeric VHH-IgG lines were sowed on kanamycin selection, and the plates were examined 3 weeks later, plates with 100% resistant plants were indicative of homozygous lines.

Piglet villi binding inhibition test

To determine if the plant made antibody inhibit the $F4^+$ETEC bacterial binding to the gut, an *in vitro* test was performed in accordance to the protocol described by Coddens *et al*. (Coddens *et al.*, 2009). In summary, the intestinal gut villi were obtained from euthanised pig, fixed (160 mM Krebs-Henseliet buffer, pH 7.4 containing 1% (v/v) formaldehyde for 30 minutes at 4°C) and a sample was tested for the presence of the receptor by incubation with $F4^+$ETEC strain. Such receptor positive villous enterocytes were then preserved in Krebs-Henseliet buffer at 4°C for further use.

The wild type F4$^+$ETEC strain GIS26 (serotype o149:k91:F4ac, LT+, ST+, STb+)(Cox and Houvenaghel, 1993) was cultured on brain heart infusion agar plates (Oxoid, Basingstoke, Hampshire, England) at 37°C for 18 hours and the cells were collected from the plate by gently flushing with approximately 3-4 ml of 1% (w/v) *D*-mannose in PBS (PBSM). Addition of mannose prevents the bacterial adhesion mediated by type 1 pili. OD of these cells was immediately measured at wavelength of 600 nm, where an OD of 1 corresponds to 10^9 bacteria per ml. Then, $4x10^8$ bacteria were incubated with either seed extract bearing anti-F4$^+$ETEC antibodies or purified anti-F4$^+$ETEC antibodies from seeds in a final volume of 450 µl made up by PBSM and incubated for one hour on a rotation wheel. Post 1 hour, the villi form the pig gut were washed with Kreb-Henseliet buffer, 50 µl of these villi were added to the bacteria-seed extract mixture and incubated again for 1 hour on the rotating wheel. After the incubation, the villi-antibody mixture (~20 µl) were taken and analysed by phase contrast microscopy at a magnification of 600 times. All the samples were blinded to eliminate experimental biasness while counting the number of bacteria bound to the villi surface. Randomly 50 µm length of the villi were selected and the number of attached bacterial cells in this length was counted; this was repeated for 20 such randomly chosen 50 µm length surface of the villi. The number of bound bacteria was expressed as an average bacteria attached for 250 µm length of the villi surface.

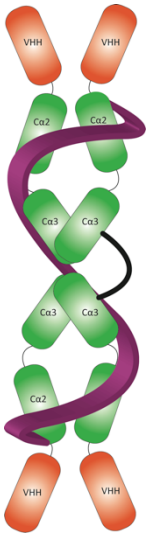
Chapter 5

Production of simplified tetravalent secretory IgA in Arabidopsis seeds for oral passive immunisation

'Strengthening the army at mucosal border security post'

Vikram Virdi, Sylvie De Buck, Annelies Coddens, Hana Hoffmeisterova, Eric Cox, Henri De Greve and Ann Depicker

H.D.G and A.D. seeded the VHH-Fc fusion strategy; V.V. conceived development of SIgA based on the VHH-IgA fusion by incorporating porcine specific J chain and secretory component. V.V developed all the clones, the transgenic plant, devised the high throughput screening method– the 2 primer multiplex PCR for transformant identification and screened the high expressing lines by the functional ELISA. H.H. helped in the screening of the 206 transformants by ELISA. S.D.B, H.D.G and A.D mentored and supervised the work. A.C. performed the *in vitro* villous adhesion test. E.C provided consultation. V.V. and A.D coordinated the project and V.V wrote the chapter.



Abstract

Mucosal surfaces are one of the chief portals for pathogen entry. The predominant protective antibody at mucosal surfaces is secretory IgA (SIgA). Topical application of recombinant SIgA antibodies at the mucosal surface can offer immediate passive mucosal immunity, which could be of vital importance in prophylaxis against many diseases for which there are no effective mucosal vaccines. However, recombinant production of therapeutic SIgA antibodies has been difficult primarily due to their complex hetero-decameric structure of ~400 kDa, hindering SIgA's clinical application. We hence conceived a novel strategy to synthesise customised and simplified forms of SIgA, henceforth abbreviated as sSIgA. These sSIgA antibodies were expressed in seeds of Arabidopsis with the aim to provide proof of concept for later cost effective bulk SIgA production in seed crops. This was achieved essentially by using the antigen binding domains from heavy chain only camelid antibodies (VHH) (~15 kDa) grafted to the fragment crystallizable (Fc) part of an IgA, such that the simplified SIgA would be a hetero-hexameric complex with molecular weight of ~230 kDa.

For pilot construction of such novel sSIgA antibody, previously isolated four VHHs against the porcine pathogen— F4 fimbriae bearing enterotoxigenic *Escherichia coli* ($F4^+$ETEC) were used. These VHHs were fused to the Fc region of porcine IgA^b, and together with porcine secretory component and porcine J chain cloned in seed-specific expression cassettes and simultaneously co-transformed into Arabidopsis plants.

Our results show that by using triple co-transformation strategy, plants expressing the three intermediate forms of SIgA, i.e. monomeric IgA (mIgA), dimeric IgA (dIgA) and secretory IgA can be selected from the pool of primary transformants. These transformed plants expressed functional, assembled sSIgA, dIgA and mIgA antibodies up to 1% of total soluble protein (0.2% of seed weight). Moreover these seed made IgA based antibodies effectively prevent $F4^+$ETEC attachment to porcine gut villous enterocytes *in vitro*.

Using the same strategy disease specific host compatible simplified SIgA antibodies can be produced as cost effective therapeutics for human and

veterinarian applications.

Introduction

Most of the pathogenic invasions start at the mucosal surface, which is an extensively vast surface lining external orifice of the body; namely the gastrointestinal tract, urogenital tract, and respiratory tract surfaces (Macpherson *et al.*, 2012; Strugnell and Wijburg, 2010). The total mucosal surface area is about 200 times larger than the total surface of the skin (in humans ~400m^2)(Brandtzaeg, 1995; Corthésy, 2002). Protection at this vast surface, by preventing the colonisation, entry and invasion of pathogens can bar the elicitation of infection. This protection is provided by a non-specific component (inclusive of sticky mucus, enzyme like lysozymes and other molecules including lactoferrins and defensins) together with a pathogen-specific mucosal immunity component (Corthésy, 2002). In case of many pathogens, the specific mucosal immunity is absolutely necessary to combat infections, for example *Vibrio cholera* infection in humans (Czerkinsky and Holmgren, 2010). In mammals (and birds) pathogen-specific mucosal immunity is offered at the mucosal surface predominantly through its arsenal of specific secretory IgA (SIgA) antibodies, hence no doubt that the SIgA are the most predominant immunoglobulin isotypes at the mucosal surfaces; being a part of the 'first line of defence' (Johansen *et al.*, 2000; Macpherson *et al.*, 2012; Wycoff, 2005).

Most vaccines are administered parenterally and fail to provide mucosal immunity, only a hand few like influenza and polio vaccine in humans are known to elicit an effective mucosal immune response (Corthésy, 2003; De Magistnis, 2006; Holmgren and Czerkinsky, 2005). Even though mucosal immunity is much needed, the lack of knowledge and understanding of the mucosal immune system, the unavailability of safe mucosal adjuvants and issues of delivery at the mucosal surface are some of the reasons for the difficulty and delay in mucosal vaccine development (Czerkinsky and Holmgren, 2010). With passive immunisation strategies, one can circumvent the priming of the immune system and provide instant protection (Li and Zhu, 2010; Naz and Rajesh, 2004). Application of disease specific SIgA antibodies topically at the mucosal surface

can strengthen the defence borders and prevent infection of the specific pathogen. The bottleneck in this approach is the manufacturing of disease specific SIgA (Corthésy, 2002).

Secretory IgA is a hetero-decameric complex secreted by the epithelial cells of the mucosal surface, typically consisting of 4 heavy chains, 4 light chains, the joining (J chain) and the secretory component (SC) (Goldsby *et al.*, 2003). The production of such SIgA *in vivo* requires orchestrated functioning of two different cell types. The plasma cells beneath the epithelium (in the *lamina propria*) produce the polymeric IgA (pIgA), which is a composite of two IgA monomers joined tail-to-tail by the J chain. Thus this is a dimeric tetravalent molecule, with 4 fragment antigen-binding domains (Fabs). The pIgA binds to the polymeric immunoglobulin receptor (pIgR) present on the basal surface of the epithelial cells (Figure 5.1, a)..

After binding the pIgR, the pIgA is internalized, and transcytosed to the apical surface where the receptor cleaves off and the previous extracellular domain is now known as the secretory component (SC). The SC then winds on to the pIgA, the entire assembly thus formed is called secretory IgA (SIgA) and is secreted at the apical luminal surface of the columnar epithelium[14] (Figure 5.1 a and b)(Corthésy, 2002; Strugnell and Wijburg, 2010)

Despite the intimidating complexity of the SIgA molecule, the benefit of SIgA in mucosal passive protection has attracted a lot of attention for its recombinant production. Initial breakthroughs were SIgA molecules produced in test tubes by combining purified pIgA from hybridoma cell lines with recombinant secretory component (Crottet *et al.*, 1999; Lullau *et al.*, 1996; Rindisbacher *et al.*, 1995). This process was later refined by stable transfection of the human secretory component (SC) in myeloma cell expressing mouse-human chimeric pIgA (Chintalacharuvu and Morrison, 1997). The importance of using a single cell was reaffirmed by many groups; following which, two of them successfully produced milligram amounts of SIgA in CHO (Chinese hamster ovary) cells using state of

[14] Animation of SIgA biosynthesis and secretion at mucosa surface can be found at http://www.neptunemediaworld.com/proed.htm

the art co-transfection techniques (Berdoz *et al.*, 1999; Johansen *et al.*, 1999). In this approach, all the elemental components were derived from human cDNA libraries with the exception of the variable regions, which were cloned from murine hybridomas (Figure 5.3). In spite of these commendable developments in the field, the inherent complexity of the molecule poses difficulty in producing cost effective SIgAs in cell cultures. This has been by far the major hurdle in realizing the full potential of SIgA for clinical use (Corthésy, 2002) both in human and domesticated animals.

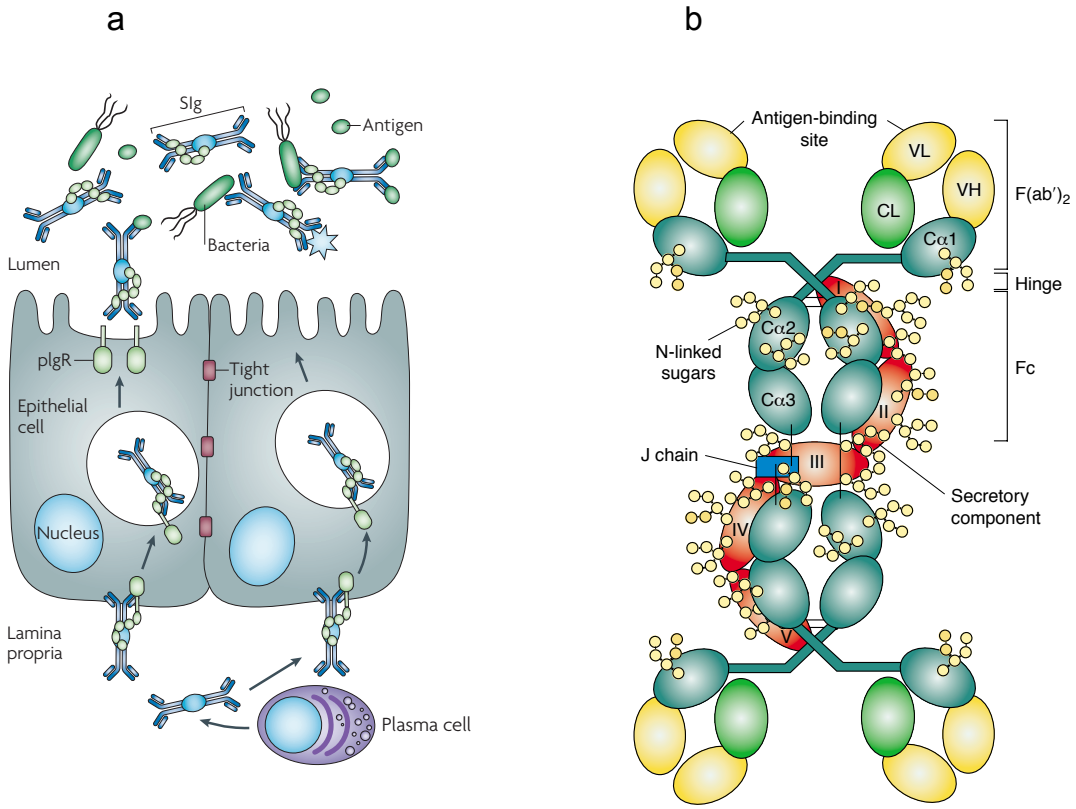


Figure 5.1: Secretory IgA: a complex heterodecameric antibody that provides protection at the mucosal surface. (a) The plasma cells in the *lamina propria*, produce J chain bound dimeric IgA (dIgA) which bind to the polymeric immunoglobulin receptor (pIgR) on the basal surface of the columnar epithelium cells, the complex is internalised, transcytosed via vesicular trafficking, the pIgR cleaves off as secretory component that wraps around the dIgA and thus formed secretory IgA (SIgA) that is secreted at the luminal mucosal surface. Fig. 5.1,a– also shows the process of immune exclusion through which the attachment of infectious agent (here a bacteria) is inhibited by SIgA. (b) A detailed schematic structure of a conventional IgA2m secretory antibody in humans. Two heavy and two light chains form a monomer, two such monomers are joined together by the J chain (blue block) via disulphide bridges across one of the heavy chain tails; the secretory component (red) with its 5 domains (roman numbers) wraps around the dIgA molecule via covalent and non-covalent bonds. The N-linked glycans are indicated by the yellow antennary structures. The paired fragment antigen-binding domains- $F(ab')_2$, the hinge, the fragment crystallizable -Fc region, the J chain, and the constant Cα1, Cα2 and Cα3 domains are labelled. Figure 5.1 a -reused from Strugnell and Wijburg, 2010 and b from Corthésy, 2002.

The only SIgA to successfully complete human clinical trials is the plant (tobacco) made anti-dental caries SIgA antibody called –CaroRx®, which is now approved for use in the European Union (Paul and Ma, 2011). This was produced by stable transformation of the heavy chain, light chain, J chain and SC in individual tobacco lines, followed by successive crossing of the selected final transformant lines. Firstly, by crossing the heavy chain and light chain, the monomeric IgA (mIgA) could be produced, crossing of this daughter line with J chain expressing tobacco line led to production of dimeric IgA (dIgA) and further crossing of this dIgA line producing plant with the one expressing SC led to a SIgA producing plant (Ma *et al.*, 1995). The success of this CaroRx® antibody is an evidence for the capacity of plants to stably produce complex SIgA, which are firstly- functional in preventing the disease, and secondly- can be scaled up for bulk production. However the *in planta* production strategy as described, is a lengthy process. Co-transformation of all the elements, i.e. IgA- heavy chain, -light chain, J chain and SC would help in shortening the time required for developing SIgA expressing primary transformants (Wycoff, 2005). Co-transformation can be achieved by either cloning the respective elements in individual plasmids (4 T-DNAs); or stacking of the elements one after the other in a multi gene expression cassette (1 T-DNA).

However at the heart of all these expression strategies, be it in mammalian or in plant systems, the daunting complexity of the SIgA prevails. Not only the transformation and expression of 4 elements, but also their assembly is demanding for the cellular machinery of the heterologous expression system. As a novel solution to this we envisaged engineering of a comparatively simplified SIgA like antibody but maintaining all advantages of a traditional SIgA. This was achieved essentially by using the VHH-IgA fusion strategy to build the monomeric units of the SIgA. More explicitly, instead of using the antigen binding domains formed by the paired heavy and light chain (the Fab ~55 kDa), we opted for the antigen binding Nanobody® (~15 kDa) which is the variable domain of a heavy chain only antibody of camelids (VHH) (summarized in Figure 5.3). This substitution also eliminated the necessity of light chain and light chain binding Cα1 domain (the first conserved domain of the α heavy chain), thereby simplifying the otherwise 400 kDa SIgA complex molecule to about 230 kDa simplified SIgA (sSIgA). Along with the simplification of the molecule, the transformation would need just three elements i.e. the VHH-IgA, the J chain, and the SC, thus also easing the transformation. Using the strategy disease-specific

simplified lightweight monomeric IgA (mIgA) with paired VHH-IgA (Figure 5.2, a), dimeric IgA (dIgA) with 4 VHH-IgA chains + J chain (Figure 5.2, b) and the sSIgA with 4 VHH-IgA chain + J chain + SC can be produced (Figure 5.2, c).

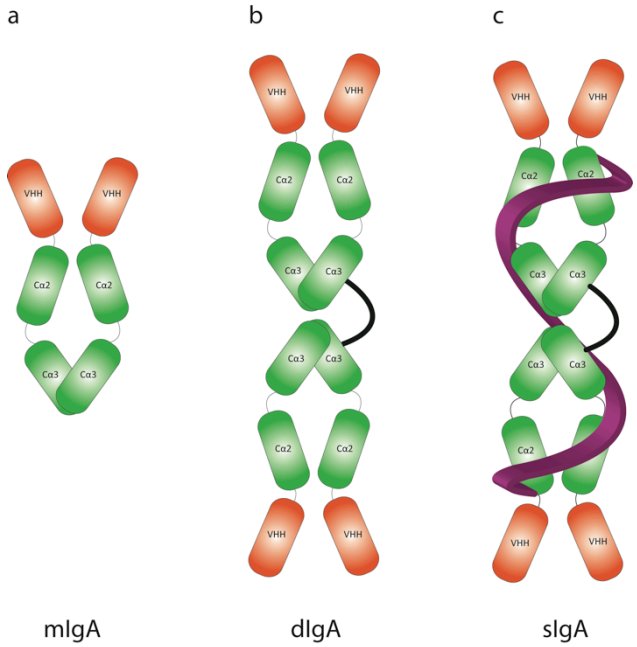


Figure 5.2: Schematic representation of the simplified IgA molecules where the VHH (red) fused to the porcine IgA^{b} (green) with a short hinge forms a divalent IgA monomer (mIgA) (a) via interchain disulphide bonds (not shown); further via incorporation of J chain (black) two such mIgA bound tail-to-tail form a tetravalent (4 VHHs) dimeric IgA (dIgA) (b); while a simplified secretory IgA (sSIgA) is formed when the secretory component (purple band) wraps around the dIgA complex (c). Abbreviations: Cα2, Cα3 are the constant domains of porcine IgA heavy chain.

To provide a proof of concept for the product as well as efficacy of such sSIgA in preventing disease, we used four previously isolated VHHs against the FaeG adhesin of the F4 fimbriae bearing enterotoxigenic *Escherichia coli* (F4^{+}ETEC) (Chapter 4). F4^{+}ETEC cause post-weaning diarrhea (PWD) in young piglets, and the disease leads to heavy economic losses to the porcine rearing industry (Amezcua *et al.*, 2002; Hong *et al.*, 2006). Piglets from birth until the weaning age are most likely protected from the infectious ETEC by the milk derived maternal antibodies against ETEC (Porter *et al.*, 1970; Riising *et al.*, 2005; Wilson and Svendsen, 1971). On weaning this passive protection at the gastric surface is lost, hence they fall prey to F4^{+}ETEC infection. Oral administration of anti-ETEC antibodies after weaning in feed, is suggested to prevent the disease (Marquardt *et al.*, 1999; Niewold *et al.*, 2007; Yokoyama *et al.*, 1992). Thus this disease in piglet model is interesting for later evaluation of efficacy and stability of sSIgA on

oral passive immunization in a challenge experiment.

With this aim we grafted the 4 anti-F4$^+$ETEC VHHs on the Fc fragment of porcine IgA^b, and co-expressed it with porcine J chain and porcine SC, in the seeds of the model plant *Arabidopsis thaliana* (Figure 5.3). The seed production platform is advantageous in this disease model, as it would also provide for a delivery system for administration of sSIgA in feed. In addition, the seed expression system is preferred for its ability to produce abundant amount of recombinant protein in a confined space (high concentration) and its merit of convenient storage at room temperature enables decoupling of the harvesting and other downstream processing of the recombinant molecule (Morandini *et al.*, 2011; Peters and Stoger, 2011).

Here we present the details of our findings demonstrating the feasibility of producing assembled, functional, simplified sSIgA molecules in seeds. The results obtained in each of the successive steps are described in the following order—starting from designing the construct, transformation, selection, characterisation, *in vitro* efficacy and finally the quantification of efficacious molecules.

Since this is the first ever description of production of simplified sSIgA, while discussing the results we suggest prospects for future developments and optimisation of the current strategy, with an aim to obtain higher accumulation of assembled customized sSIgA.

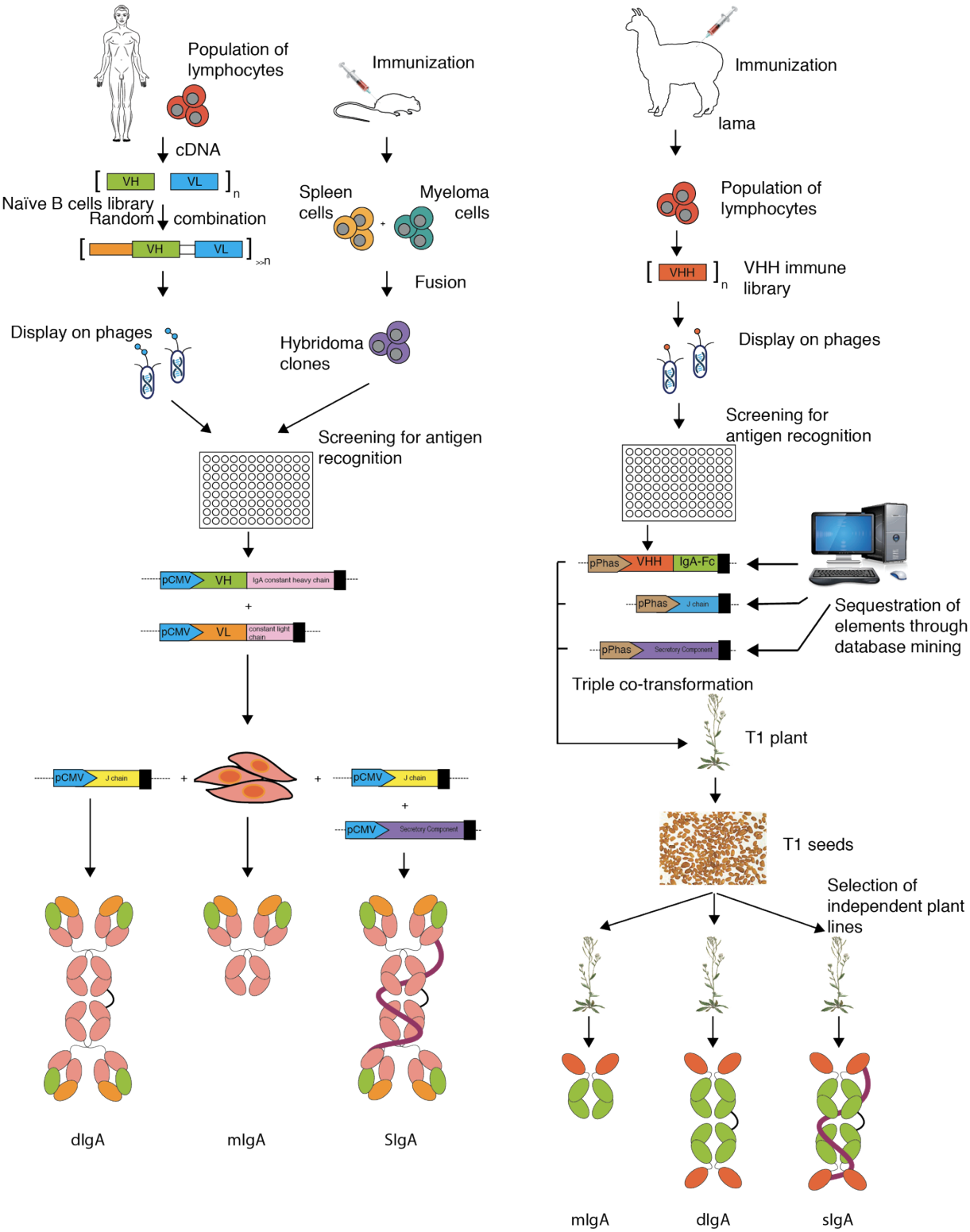


Figure 5.3: Comparative schematic of current strategy used for secretory IgA (SIgA) production in mammalian cells- either from naive human B cell library or murine hybridoma clones (left panel) versus, in seed production of pathogen specific simplified secretory IgA (sSIgA) from lama immune library. The mammalian SIgA expression strategies (left) is summarised and adapted from Corthésy, 2002.

Results and Discussion

In silico sequestration of elements for porcine sSIgA construction and cloning

Instead of the traditional PCR based sequestration of the immunoglobulin element from the cDNA library, and then replacing the regulatory elements by molecular cloning techniques (Corthésy, 2002), we opted for *in silico* analysis and sequestration followed by immediate chemical synthesis of the desired coding sequences.

Porcine J chain

The amino acid sequence of the porcine J chain is not defined, however there is a very high degree of conservation noted in the J chain throughout evolution, as concluded from the comparative analysis of the J chain amino acid sequences in an invertebrate like earthworm, and in higher vertebrates including amphibian-like *Xenopus* and mammals like– mouse, bovine, rabbit, horses, etc. (Johansen *et al.*, 2000; Lewis *et al.*, 2010). It was also demonstrated that the J chain of humans was interchangeable with J chain of mouse and chicken; however the affinity to bind the IgA to form pIgA decreased with higher phylogenetic distance between them (human > mouse > chicken)(Johansen *et al.*, 2000).

To isolate the porcine J chain sequence, we used the human J chain sequence (Uniprot accession no P01591) as bait, given the overall genetic similarity between humans and pigs. By a homology-based search with the human J chain bait (accession no: NP_653247), within the porcine EST (expression sequence tag) database we could identify the porcine J chain homologous sequence from the cDNA library made from porcine alveolar macrophages (accession no.: AK231006). Within this cDNA sequence using BLAST-X we could identify the putative J chain coding sequence that started from the 72nd nucleotide (ATG) until the stop codon (TAA) at the 548th nucleotide. This gene coding sequence of 476 bp was then defined as porcine J chain.

The deduced amino acid sequence was aligned with mammalian J chain sequence of human (p01591), mouse (p01592), rabbit (p23108) and bovine (Q3SYR8) available in UniProt database, as reference (Figure 5.4). We noted 80.62%

identity between the porcine J chain and the human J chain sequence. The 71st asparagine residue (numbering according to human J chain), which is the acceptor site for N-linked glycosylation was conserved in all sequences analysed (Figure 5.4) and also present in the porcine J chain and predicted to bear N-link glycosylation as analysed with NetNGlyc 1.0 (with potential of 0.7061, and 9/9 jury agreement)[15] (Gupta *et al.*, 2004). The position of the 8 cysteine residues for the inter- and intra-chain disulphide bonds and the domains implicated for SC binding were highly conserved in the sequence analysed (denoted in Figure 5.4 in accordance with (Lewis *et al.*, 2010)).

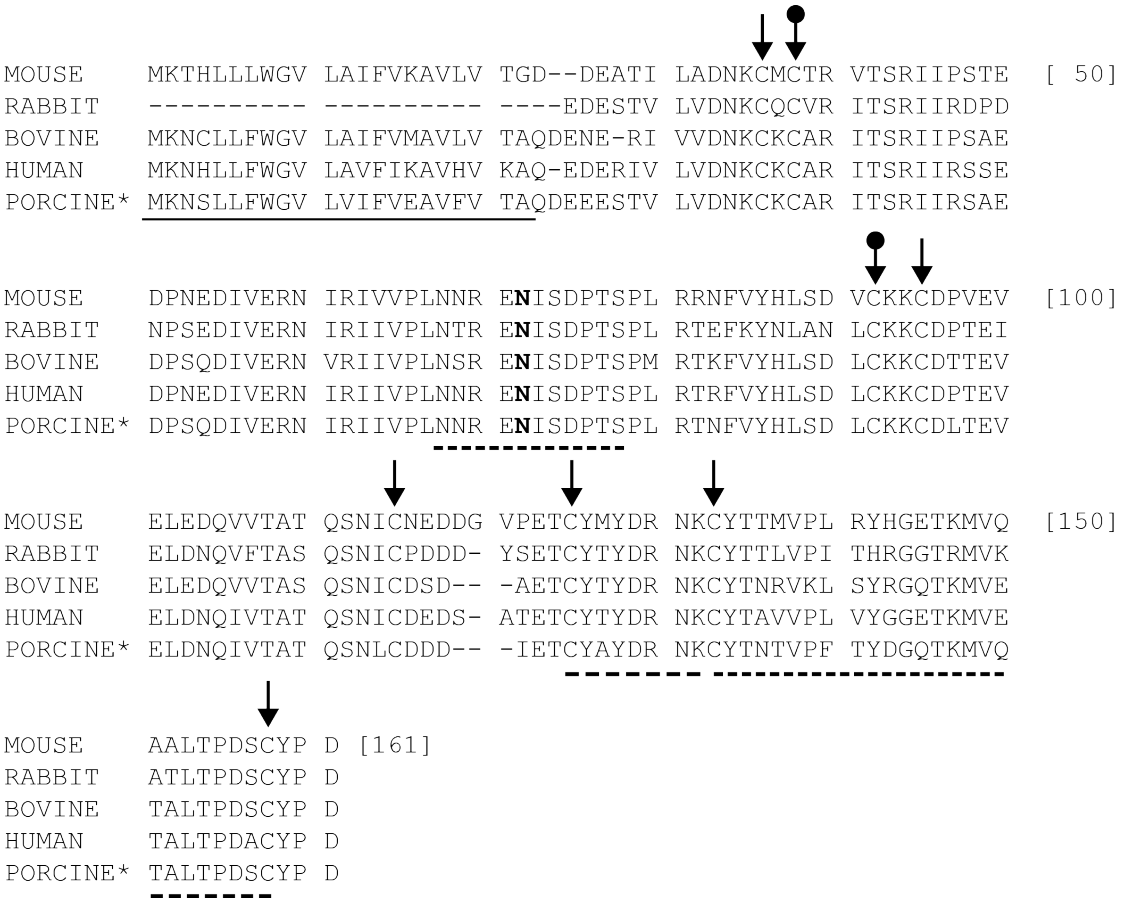


Figure 5.4: Deduced amino acid sequence of porcine J chain aligned with J chain sequence of human, mouse, rabbit and bovine. The 8 conserved cysteine residues involved in disulphide bridges are indicated with arrows, 2 among these involved in interchain bonds with IgA heavy chains are indicated with arrows bearing a circular tail. The porcine signal peptide is underlined with a solid line, while the regions associated with binding to the secretory component are underlined with a dotted line. The N-linked glycosylation site in human was found to be conserved in all the sequences analysed (bold faced), this residue was also predicted as putative N-link glycosylation site in porcine J chain sequence.

[15] http://www.cbs.dtu.dk/services/NetNGlyc/

Further, from the alignment and by using the signal peptide predictor (SignalP 3.0) (Bendtsen *et al.*, 2004) the native signal peptide of J chain was identified (underlined in Figure 5.4) and replaced with the signal peptide of 2S2 Arabidopsis seed storage protein. Thereafter, the codon usage in the entire coding sequence was optimised according to the codon usage of the major storage proteins in Arabidopsis seeds. The nucleotide coding sequence for the endoplasmic reticulum retention signal (KDEL) was added to the (C-terminal) end followed by a stop codon and the DNA was chemically synthesised within the attB1 and attB2 for Gateway cloning (Invitrogen).

Porcine Secretory Component

The secretory component, which is the resultant product of proteolytic cleavage of the polymeric immunoglobulin receptor (pIgR), is either found as free SC form or associated with the dimeric IgA. Structurally the pIgR has 5 domains that are connected to a transmembrane region by a linker peptide (Figure 5.5); the cleavage takes place somewhere in the linker region, after the 5th domain, and these 5 extracellular domains wrap around the dIgA.

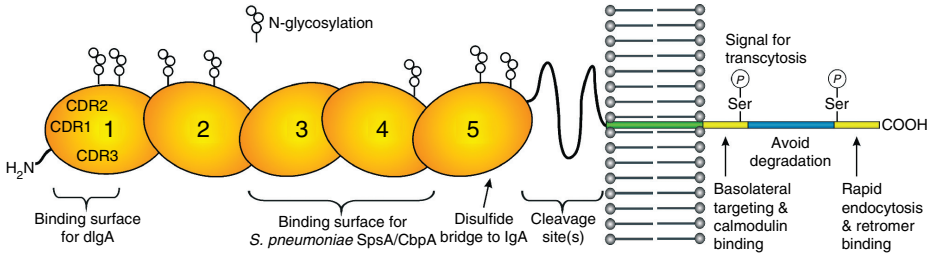


Figure 5.5: Schematic representation of the polymeric immunoglobulin receptor (pIgR) bound to the basal surface of the columnar epithelial cells of the mucosal surfaces. The receptor has 5 domains with immunoglobulin-like folds, and is heavily glycosylated. Figure reused from Kaetzel, 2005.

The sequence of the porcine SC was hence derived from the published sequence of porcine pIgR (Kumura *et al.*, 2000)(accession no.: NM_214159, UniProt accession no. Q9N2H7). The endogenous signal peptide of pIgR was determined (supplementary Figure 5.S1) as described for the J chain, using the signal prediction tool (Bendtsen *et al.*, 2004) and then *in silico* replaced by the signal peptide of the 2S2 seed storage protein of Arabidopsis seeds at the N-terminal

end. However the cleavage site and hence the exact length of the free porcine SC is not determined yet, moreover, only the length of the free SC of humans is known (Kaetzel, 2005), but there is a discrepancy in the published C-terminal residue. Free human SC from pooled colostrum, suggested of having ragged C-terminal end varying from Ala550 to Lys559 however Ser552 was the prominent end (Eiffert *et al.*, 1984), while in another study from a single woman, Arg585 was determined as the last residue of human SC (Hughes *et al.*, 1997). It is difficult to accurately guess the cleavage site of the porcine SC since there is little sequence identity in the cleavage linker peptide of different species. Over all given that the first 5 domains of the porcine pIgR are important for wrapping around the dIgA, we selected and defined the first 579 aa length-spanning region until the 5th domain after the signal peptide (corresponds to the 585 aa of human SC) as porcine SC. The nucleotide sequence of this porcine SC (1737 bp) together with the plant signal peptide at the 5' end (N-terminal end) and a KDEL retention signal at the 3' end (C-terminal end) was codon optimised and chemically synthesised with attB1 and attB2 flanking sequences.

Porcine IgA Fc

The VHH-IgA fusion forming monomeric IgA is the third element in our sSIgA strategy. The porcine IgA has three allelic forms IgA^a, IgA^b and the recently describes IgA^c which basically occurs due to alternative splicing (Brown *et al.*, 1995; Kunhareang *et al.*, 2009). Of these, the porcine IgA^b has the shortest hinge with just 2 amino acid, making it also one of the smallest hinge reported yet among the IgA of mammals and birds (Brown *et al.*, 1995). Just like human IgA2, which has a short hinge and lacks the site for bacterial IgA proteases, the occurrence of short hinge in pig is suggesting to be resistant to proteolysis (Brown *et al.*, 1995). Hence the IgA^b was chosen as the fusion partner for VHH as it would be more fit in the gastric tract and thus suitable for oral passive immunisation; though a short hinge usually implies a rigid, inflexible hinge. The sequence of the hinge-Cα2-Cα3 was retrieved from the NCBI nucleotide database (accession no. U12594, from the porcine IgA^a sequence, the coding frame of short hinge starts from the 312th base pair). The codon usage in this nucleotide sequence was then optimised to Arabidopsis seeds and the previously isolated 4

anti-F4⁺ETEC VHHs viz. V1, V2, V3 and V4 (Chapter 4) were grafted onto the pig IgA^{b} hinge-Cα2-Cα3 domains (Figure 5.2, a); the resultant fusion antibodies were called V1A, V2A, V3A, V4A respectively. We also grafted the codon optimised version of V1 i.e. V1c and V4 i.e. V4c onto the porcine IgA, however this construct did not bear any difference in expression, or any of the parameters measure hence they are not elaborated the difference in this chapter.

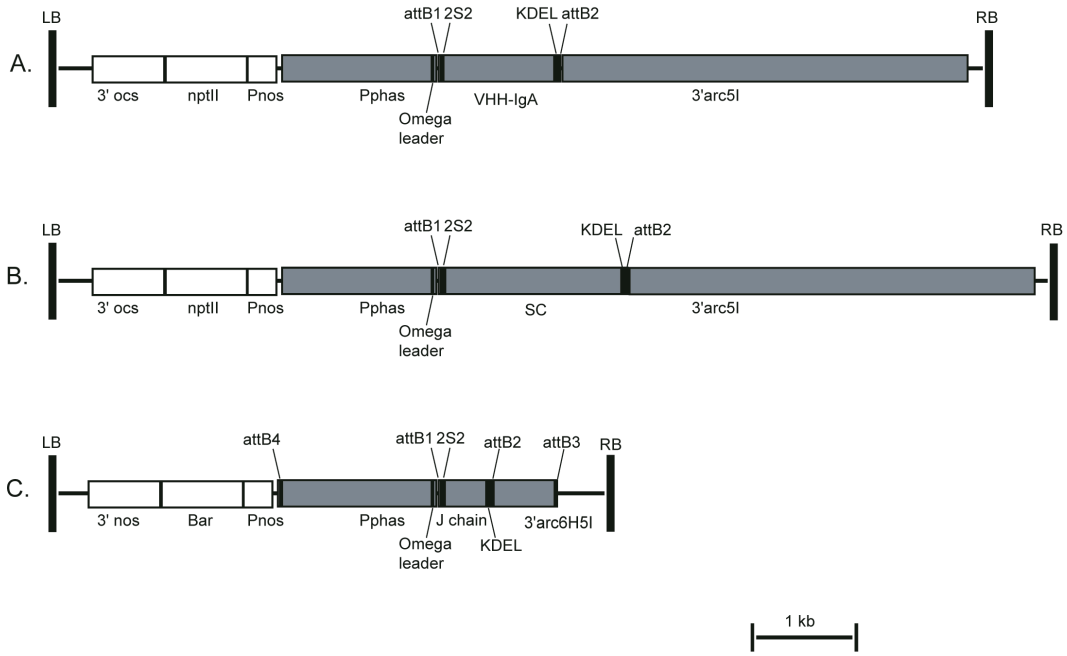


Figure 5.6: Schematic representation of the T-DNA constructs for in seed expression of anti-F4⁺ETEC secretory IgA antibodies. Abbreviations: LB- left border; RB- right border; VHH-IgA-fused coding sequence of the VHH-IgA; SC-porcine secretory component, J chain-coding sequence of the porcine J chain; Omega leader- 5' tobacco mosaic viral UTR; 2S2 - signal peptide sequence of the 2S2 seed storage protein; KDEL- endoplasmic retention motif; attB1, attB2, attB3 and attB4 - Gateway recombination sequences (Invitrogen); Pphas- Phaseolin promoter; 3'arc5I-3' arcelin terminator (4100 bp); 3'arc6H5I- 600 bp long 3' arceline terminator bearing regulatory sequence; *nptII*- neomycine phosphotransferase II gene; Pnos- nopaline synthase gene promoter; 3'ocs- octopine synthase terminator; *bar*- phosphinothricin herbicide resistant gene; 3'nos-nopaline synthase terminator.

The KDEL endoplasmic retention signal was attached to all the elements, to facilitate *in situ* assembly of the sSIgA molecule (Figure 5.6). Using the gateway recombination technology (Invitrogen) V1A, V2A, V3A, V4A and SC were cloned into pPhasGW expression vector (Morandini *et al.*, 2011), under the control of the seed storage β-phaseolin promoter and arcelin (4100 bp) terminator

sequence, bearing the kanamycin resistance on the T-DNA. While the J chain was cloned together with the β-phaseolin promoter and short arcelin (600 bp) terminator (De Wilde, 2012) via the Multisite Gateway technology into the pBm43GW,0 (Karimi *et al.*, 2005) plasmid, the T-DNA of which bears the *bar* gene that confers for phosphinothricin (PPT) resistance, enabling selection of J chain expressing plants. In order to produce 4 different SIgA, each of the VHH-IgA, i.e. V1A, V2A, V3A and V4A were co-transformed with the J chain and SC.

Production and selection of functional- monomeric, dimeric and secretory IgA antibody expressing plants from pool of co-transformants

Ten Arabidopsis (Col 0) plants were transformed by floral dip method, for each of the 4 sets of triple co-transformation (V1A+SC+J; V2A+SC+J; V3A+SC+J; V4A+SC+J) to produce different formats of IgA antibodies bearing 4 different antigen binding domain.

a.

	mIgA	dIgA	sIgA
V1A	10 (0.1%)	17 (0.17%)	4 (0.04%)
V2A	7 (0.07%)	6 (0.06%)	1 (0.01%)
V3A	12 (0.12%)	3 (0.03%)	5 (0.05%)
V4A	21 (0.21%)	5 (0.05%)	1 (0.01%)

b.

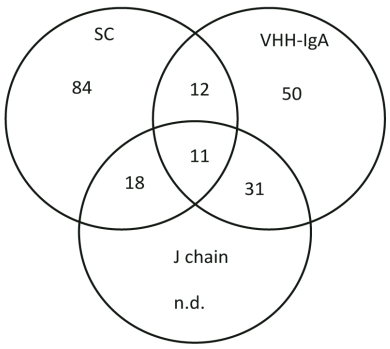


Figure 5.7: Identified monomeric-IgA (mIgA), dimeric-IgA (dIgA) and simplified secretory IgA (sSIgA) expressing lines after triple co-transformation of VHH-IgA, J chain and porcine secretory component (as determined by 2PM-PCR). The table (a) indicated the number of primary transformants from each of the 4 sets, expressing single element, VHH-IgA (mIgA); two elements, VHH-IgA and J chain (dIgA); or three elements, VHH-IgA, J chain and SC (sSIgA). The transformation frequency is indicated in parenthesis. The Venn diagram (b) shows the classification of the 206 primary transformants obtained on co-transformation. Where n.d. stands for not determined.

From each transformed plant ~1000 T1 seeds were first sown on kanamycin bearing Murashige and Skoog medium, and a total of 206 lines were identified (86 for the V1A set, 43 for the V2A set, 51 for the V3A set and 63 for the V4A set).

Leaves from these 206 primary transformants were then placed on callus induction medium with phosphinothricin (PPT) selection, induction of healthy callus from 60 of these 206 T1 leaves identified J chain expressing plants (25 of the 86 from the V1A set, 6 from the 43 of the V2A set, 16 from the 51 of the V3A set and 13 from the 63 of the V4A set were identified for the presence of J chain). However this does not allow distinction between VHH-IgA and SC expressing plants since both T-DNAs confer kanamycin resistance. Further using a multiplex PCR set up with just 2 primers (2PM-PCR) compementary to the regulatory elements on the T-DNA, the presence of the respective transformed genes were determined in a single reaction per primary transformant plant. From these results, T2 plant transformed with different permutation of the three T-DNA were classified (Venn diagram, Figure 5.7); the most interesting of these combinations were monomeric transformant plants– with only VHH-IgA bearing T-DNA, dimeric IgA plants– with IgA and J chain bearing T-DNAs and the sSIgA transformant plants– with all the three T-DNA inserts bearing VHH-IgA, J chain and SC coding sequence (Venn diagram, Figure 5.7). [Nomenclature: on classifying the plants into the three groups, a small letter 'm' for mIgA lines, 'd' for dIgA lines, and 's' for secretory IgA lines was written before the name of the individual transformant plant]

Thus unlike previous plant made SIgA (Ma *et al.*, 1995) as introduced earlier, our method of co-transformation demonstrates fast production and isolation of lines expressing all three intermediate forms of sSIgA antibodies. The 2PM-PCR approach especially facilitated the fast screening. Immunodot blot as an alternative method of fast screening was ineffective as it led to high false positive results (data not shown). The results of 2PM-PCR on the contrary had no false positive results, and a few false negatives. Overall the screening results were more than 95% in agreement with results observed from plants grown on selection plates and callus induction test. For the interesting population identified among the pool of 206 primary transformants, the presence of all the respective elements could be confirmed with SDS-PAGE separated immunoblots. Hence 2PM-PCR approach could be used for future identification of sSIgA elements.

Stacking of all the three sSIgA elements in tandem on one T-DNA might further speed up the selection process, as it would eliminate the need of such multiplex PCR, and additionally would also ease in identifying single locus insertion lines. However, since each of these triple tandem elements bearing transformants will ideally express all their elements, the strategy does not allow for selection of individual transformant lines exclusively expressing mIgA (VHH-IgA) or dIgA (VHH-Iga +J chain). Thus for example, for production of exclusive dIgA expressing line new expression cassettes with VHH-IgA and J chain in tandem will have to be cloned. Depending on the application, a suitable strategy could be used, however isolation of different lines from a pool/library might be of interest for pilot / proof of concept studies.

Selection of lines expressing high amounts of functional IgAs and characterisation of the complexes

To assess the accumulation of the assembled, functional IgA antibodies in the plants classified as mIgA, dIgA and sSIgA, we used an ELISA based approach with immobilised antigen– FaeG. The T2 seed extract from the respective plant was added into the wells, any functional IgA molecules that were bound to the antigen, were characterised further using antibodies specific against each of respective 3 elements, thus the bound functional IgA antibodies were characterised in solution. Such an ELISA based strategy is apt for determination of functional sSIgA complexes, since the SC interaction with the dimeric IgA molecule is not strictly covalent in nature. Previously Ma *et al*. also utilised a similar methodology to characterise the tobacco made SIgA (Ma *et al.*, 1995).

Monomeric IgA

Using anti-porcine-IgA polyclonal antibody, the functional monomeric IgA specifically recognising the antigen could be identified by ELISA. The overall accumulation of functional VHH-IgA was reasonably similar for all the four VHH-IgAs, i.e. the range of variation was very low (Figure 5.8) hence, among the pool of 50 monomeric VHH-IgA expressing T2 plants, 15 high expressing transformant plants were selected for further characterisation and up scaling. Of these 15, 5 were mV1A, 2 were mV2A and 4 each of the mV3A and mV4A (indicated with dotted line in Figure 5.8). Among these lines, 9 had single locus

insertion of the VHH-IgA gene and a germination frequency of more than 40%. The VHH-IgA in these 9 selected lines was analysed further by immunoblot under reducing conditions with purified standards as reference (Figure 5.8).

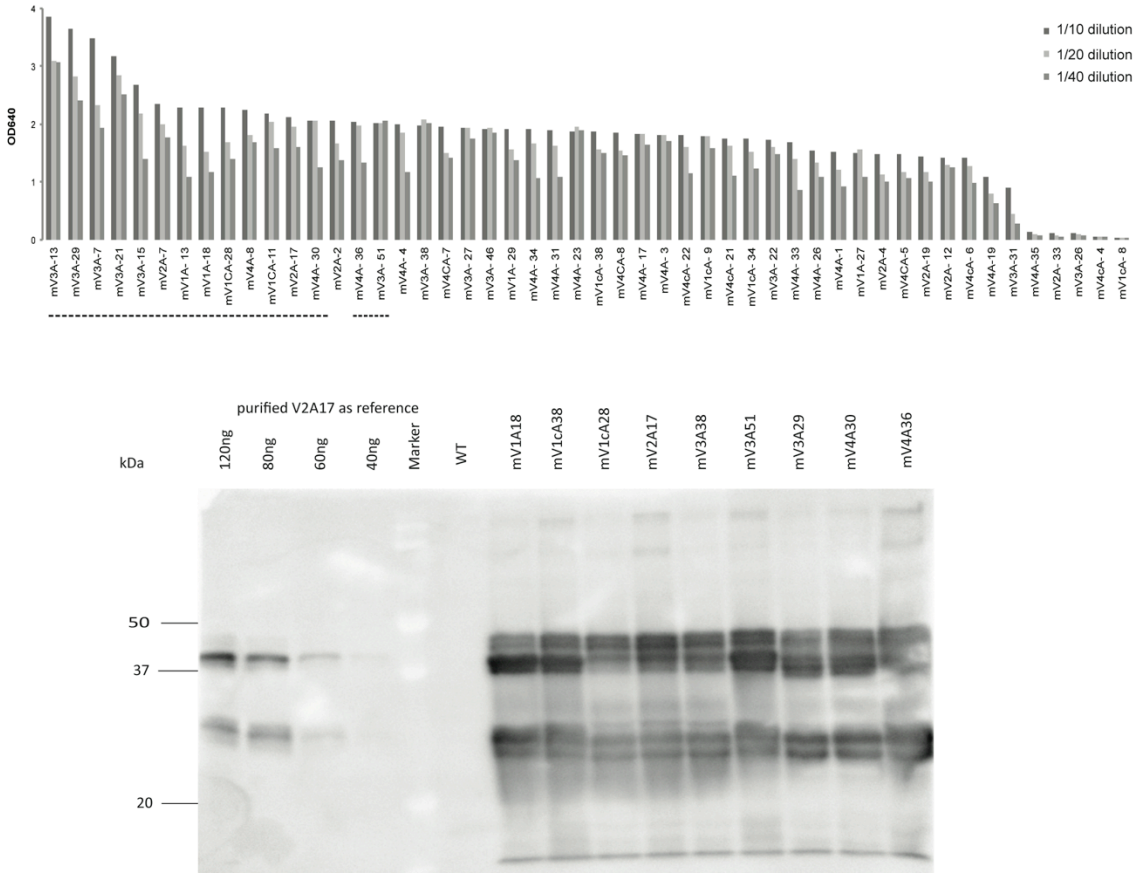


Figure 5.8: Selection and characterisation of monomeric IgA producing plant lines. The accumulation of functional mIgA as determined by ELISA in the 50 primary transformants was reasonably similar with very little range in variation, and extremely low accumulation detected in just 5 lines (top graph, extreme right samples) The OD was determined for seed extracts diluted 1/10, 1/20 and 1/40 (see key). The 15 highest expressing transformants (underlined in graph) were selected for further analysis, 9 of these has single locus insertion. Under reducing conditions (lower immunoblot) the VHH-IgA in these single locus high expressing mIgA lines showed occurrence of 4 bands about the expected molecular weight of 37 kDa and proteolitically cleaved lower molecular weight bands around 25 to 20 kDa. Each lane contained 10 µl of seed extract made by suspending every milligram of crushed seeds in 100 ml of buffer representative of identical seed weight and separated on a 12% SDS-PAGE, the individual transformant are indicated above the immunoblot, where WT stands for wild type seed extract. As reference standard, the seed made mV2A antibody was purified from mV2A17 seeds using (SSL7) affinity chromatography, and 120 ng, 80 ng, 60 ng and 40 ng of this was loaded in wells as on the left side of the marker. The anti-porcine IgA antibody conjugated to HRP was used to detect the VHH-IgA bands.

Surprisingly, instead of the expected single 39 kDa VHH-IgA band, 4 heterologous bands were seen from 37 kDa until 50 kDa (Figure 5.8), additionally lower molecular weight bands of ~20 kDa and ~27 kDa were observed. These lower molecular bands could presumably be the result of in seed proteolysis (rather than during sample preparation as higher than recommended dose of protease inhibitor cocktail was used in the sample buffer). These bands were not seen in the wild type control seed extract and were hence indicative of specific porcine IgA Fc expression and proteolysis. Duplex of heterologous protein bands are not uncommon (also reported in Chapter 4), and have been reported by our group previously on expression of IgG and IgG composite or derived fragments, these duplex bands usually result from differential glycosylated IgG heavy chain (Van Droogenbroeck *et al.*, 2007). The IgA Fc bears comparatively more putative sites for glycosylation (Figure 5.1, b and supplementary Figure 5.S2), and aberrant glycosylation in the VHH-IgA could explain the quadruplet bands. We investigated this hypothesis further, and later in this manuscript we report and discussed these findings in more detail (see section glycosylation and proteolytic cleavage).

Simplified secretory IgA

All 60 plants expressing J chain (callus induction positive) with SC and VHH-IgA, or with either of them (Figure 5.7) were analysed in two separate ELISAs with monoclonal antibodies against the SC and against the J chain together with the wild type seeds extract as negative control. In the ELISA developed with anti-SC antibody only 9 of the 15 lines (Figure 5.7) classified as putative sSIgA expressing lines by 2PM-PCR had a signal higher than the negative control (OD640 of wild type negative control was 0.04). The titre of signal in these 9 seed extracts declined with serial dilution demonstrating the specific biological relevance of the assay. Plants expressing just the VHH-IgA + J chain (dIgA), or the ones expressing SC and J chain did not give signal above the background in this anti-SC ELISA set up, signifying that only the fully assembled functional sSIgA were detected in ELISA (data not shown here). The results also gave a comparative insight into the level of accumulation of these functional sSIgA (Figure 5.9, a).

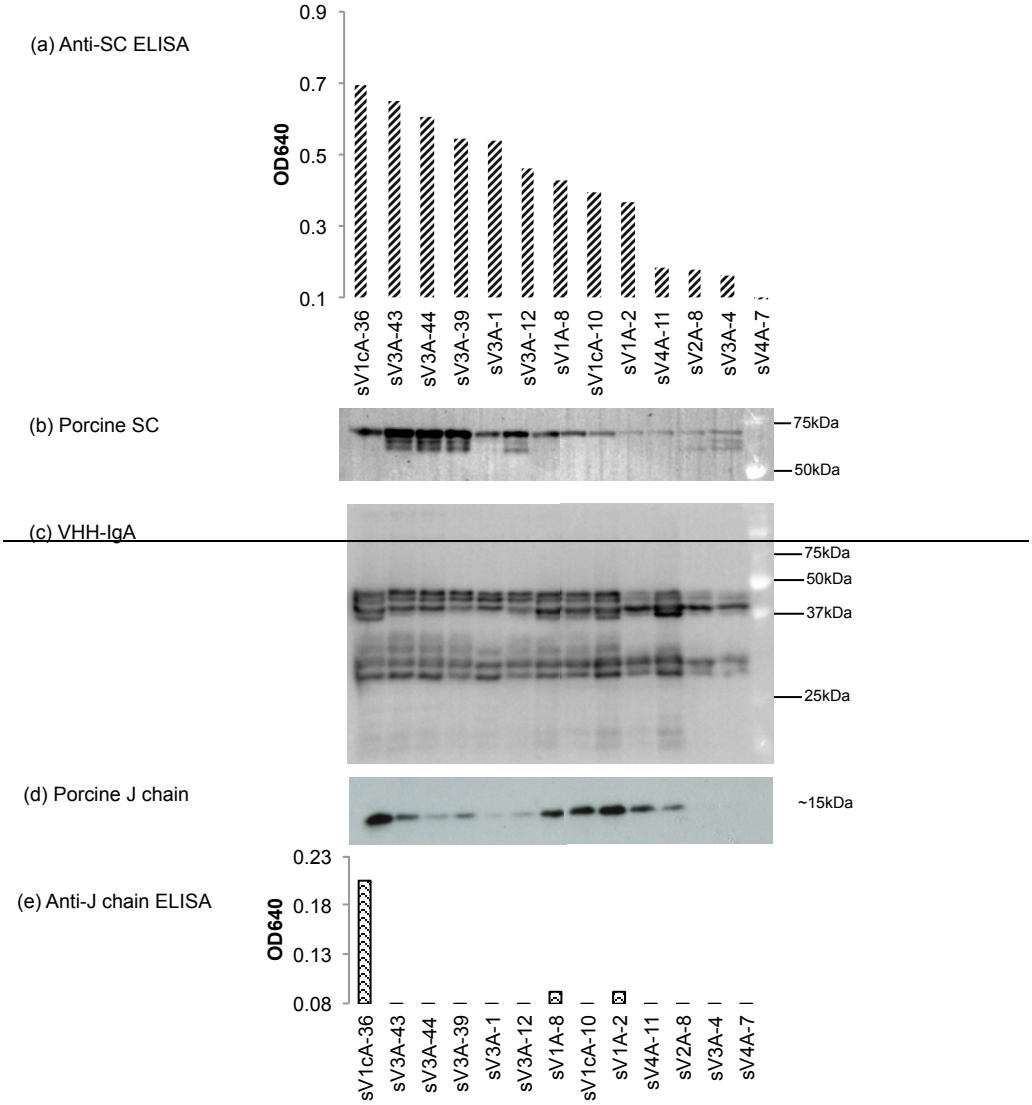


Figure 5.9: Characterising sSIgA produced in Arabidopsis seeds. The functional anti-SC ELISA (a) shows the comparative accumulation of assembled sSIgA in the 9 high expressing lines in descending order of accumulation followed by three lines that had very low signal. The same order of samples has been maintained in all the 5 panels. Three of these 9 lines also expressed functional, assembled dIgA as seen in the anti-J chain ELISA (e). The production of these assembled, functional molecules detected by ELISA depended on the individual accumulation of each of the elements– the SC (b) (detected with monoclonal anti-porcine SC), the VHH-IgA (c) (detected with polyclonal anti-porcine IgA serum), and the J chain (d) (detected with anti-J chain monoclonal antibody) as detected under reducing immunoblots. The three elements were determined from a single blot, by successive stripping of the previous detection antibody. Each lane contains 10 µl of seed extract made by suspending every milligram of crushed seeds in 100 µl of extraction buffer (i.e. each lane is representative of 100 µg of seed weight).

The ELISA with the anti-J chain antibody detected functional molecules in 3 of these 9 sSIgA lines of which sV1cA36 had a much higher titer as compared to the other two lines sV1A8 and sV1A2 (Figure 5.9, e). The SDS-PAGE immunoblot analysis under reduced condition on these 9 sSIgA-producing lines confirmed the

accumulation of J chain in all of these seeds (Figure 5.9, d). This suggests that the presence of the SC in sSIgA antibody masks the interaction of the monoclonal anti-J chain antibody in 6 of the 9 sSIgA lines, while in the other 3 lines the comparatively higher expression of J chain than the SC might have led to surplus of J chain bearing dimeric IgA (especially in sV1cA36). Thus in these 3 lines bi-species of IgA as– dIga and sSIgA antibodies were produced. The bi-species producing line sV1cA36 was also the highest producer of both functional sSIgA and dIgA antibodies among all the plants (Figure 5.9). This high expression might be the reason for the overall lower germination frequency of about 50% observed in this line. We further investigated the relative accumulation level of each of the elements of sSIgA under reducing conditions via immunoblot analysis. The results showed that indeed the expression of the SC in these 9 sSIgA lines correlates with the relative expression of assembled sSIgA determined by ELISA (Figure 5.9, a and b). The expression of VHH-IgA in all these 9 lines was reasonably similar, and was not the rate-limiting factor for assembly of sSIgA (Figure 5.9, c). The levels of J chain expressed in the 9 sSIgA lines was rather variable, and comparatively high in line sV1A8, sV1A2 and sV1cA36 which might explain the co-detection of both the sSIgA and dIgA antibody formats. Our data also implies that the molar accumulation of each of the elements in sSIgA has an effect on the final accumulation of assembled sSIgA antibodies.

Precise molar expression of each constituent element for maturation and assembly of complex heterologous polyproteins has been reported previously for complex Blue tongue virus (BTV) like particles produced in tobacco (Thuenemann and Lomonossoff, 2010). BTV is a complex of 4 different proteins; random accumulation of these 4 proteins leads to inefficient production of BTV like particles, with lots of unassembled monomers being wasted. This group showed that by fine tuning the regulatory elements the precise molar expression of each component can be achieved, which in turn led to efficient high level expression of assembled BTV like particles (Thuenemann and Lomonossoff, 2010). Similar strategies to modulate the molar expression of each constituent element by fine-tuning the regulatory elements can be tested for sSIgA production in seeds. Recently, our group reported that the longer arcelin terminator bearing 3' regulatory region (length 4100 bp vs 600 bp) correlates to

comparatively higher accumulation (De Wilde, 2012). Thus by adjusting the length of 3' regulatory sequence the rate of accumulation of each of the polypeptides chains of the SIgA can be fine-tuned in seeds.

Further, the stoichiometry of each of these elements might also influence the assembly and hence their detection in ELISA. The SC is a highly glycosylated protein when expressed in mammals both natural as well as in recombinant systems (Corthésy, 2002). The seed produced porcine SC also bear glycans, which could be detected by glycan specific stain (Figure 5.11). There was aberrant glycosylation of the SC detected in many of the plant lines, resulting in two to three glycoprotein bands ranging from ~65 kDa to ~75 kDa (Figure 5.9, b). We did not evaluate if the glycans decorating the SC or the VHH-IgA had any influence on the assembly of the complex, but it would be interesting to evaluate this in future.

Also worth evaluating is the necessity of J chain for the assembly of tetravalent simplified sSIgA within the 12 lines expressing only the SC and the VHH-IgA (Venn diagram, Figure 5.7). However, it has been stated in previous reports of SIgA production (heavy, full size conventional molecule) that the presence of J chain is necessary for the formation of SIgA in mammals (Brandtzaeg and Prydz, 1984), also in recombinant expression systems both *in planta* (Ma *et al.*, 1995) and in mammalian cells (Corthésy, 2003; Rindisbacher *et al.*, 1995). The recombinant SC does not interact with monomeric IgA that are devoid of J chain.

Dimeric IgA

The J chain ELISA detected 7 transformants expressing assembled functional dimeric IgA among the 32 plant lines identified as dIgA producing lines by PCR (Figure 5.7 and 5.10). The results as compare to controls also indicate that the J chain specifically interacts with the VHH-IgA and does not bind to the antigen FaeG in the absence of VHH-IgA (data not shown here).

Among these 7 functional dIgA producing lines, plant dV4A2 did not bear enough seeds for further analysis and propagation; the remaining 6 lines were characterised under reducing condition via immunoblot. The immunoblots developed with anti-SC, anti-porcine IgA and anti-J chain antibody confirmed

that these 7 lines did not express any SC, and the detected assembled antibody is composite entirely of VHH-IgA chains and the J chain (Figure 5.10). The VHH-IgA accumulation in all the 6 lines was reasonably similar (Figure 5.10, b). The accumulation of the functional complex (ELISA) seemingly correlated with the expression of J chain in 5 out of the 6 lines examines (Figure 5.10, a and c), the line dV3A10 showed the highest amount of J chain being expresses (immunoblot-Figure 5.10, c) but in comparison had lower signal for functional dIgA in ELISA (Figure 5.10). The pattern of abundance of the 4-glycosylation bands in the VHH-IgA of the dV3A10 lines was not similar to the other 5 lines; the proportion of the high molecular weight bands was comparatively more in this line. Though speculative, this dissimilarity might have an influence on the comparatively lower assembly of the functional dIgA, in plant dV3A10.

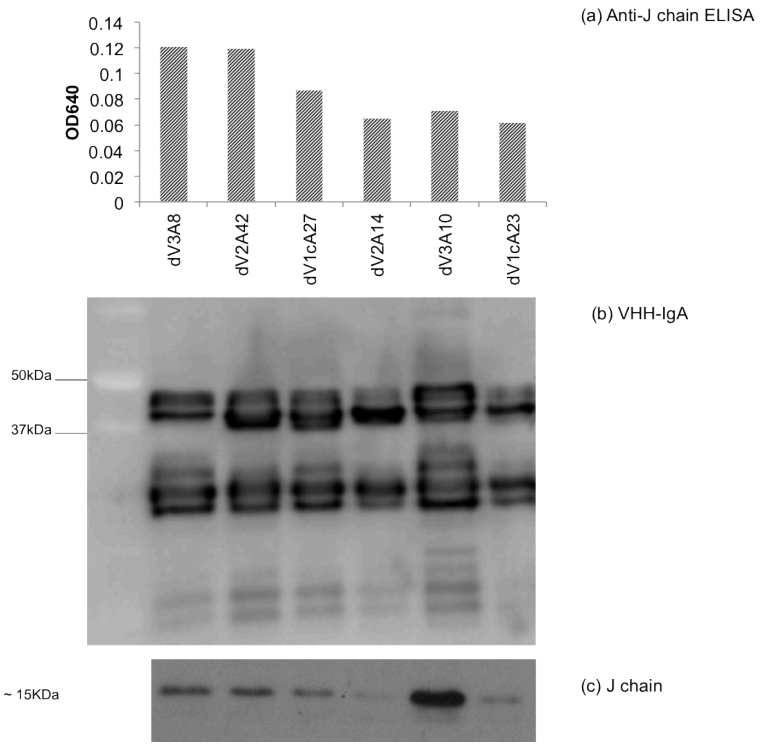


Figure 5.10: Characterising the dIgA producing seeds for assembled functional antibodies. The two parameters firstly the relative accumulation of functional assembled dIgA detected via anti-J chain ELISA (a) and secondly the corresponding accumulation of each of the two constituent elements i.e. VHH-IgA (b) (detected with polyclonal anti-porcine IgA serum), and J chain (c) (detected with anti-J chain monoclonal antibody) as determined by immunoblot under reduced conditions correlates for all but dV3A10. The two elements were detected from the same immunoblot, by successive stripping of the previous detection antibody. Each lane contains 10 µl of seed extract made by suspending every milligram of crushed seeds in 100 µl of extraction buffer (i.e. each lane is representative of 100 µg of seed weight). The bands were separated on a 12% SDS-PAGE, prior to immunoblotting.

Glycosylation and proteolitic degradation of VHH-IgA as factors affecting assembly of IgA

The VHH-IgA expressed in the Arabidopsis seeds showed the presence of 4 bands under reduced condition on immunoblots developed with anti-pig IgA polyclonal antibodies. The relative abundance of each of the 4 bands differed from plant to plant, but was invariably present in all the transformants irrespective of the co-expression of the SC and, or the J chain. In light of previous knowledge of in seed expression of IgG antibody like construct (Loos *et al.*, 2011a; Loos *et al.*, 2011b; Van Droogenbroeck *et al.*, 2007), these 4 functional (Supplementary Figure 5.S5) bands could either be the result of differential glycosylation or the proteolytically truncated version of the intact protein. Our results as elaborated below suggest that both of these reasons might contribute to the observed 4 bands.

The IgA heavy chain is heavily glycosylated and bears both N–linked and O-linked glycans in natural system, as well as when recombinantly expressed in mammalian and plant systems (Karnoup *et al.*, 2005). The N-linked glycosylation prediction of the VHH-IgA sequence assigned two asparagine residues N147 and N346 as putative N-linked glycosylation sites (supplementary Figure 5.S2)(Gupta *et al.*, 2004). The homologous position of the N346 residue is conserved in humans, gorilla, gibbon and orangutan; this residue is suggested to bear N-linked glycosylation in these species as well (UniProt accession no. P01876 (human); P20758 (gorilla); H9HA31 (gibbon); A2NVA6 (orangutan); N.B. position of N346 in VHH-IgA is identical to N340 in primate sequence analysed). On the other hand, the O-linked glycosylation prediction– Net-0-glyc software indicated a threonine residue in the framework region 4 of the VHH to be a putative O-linked glycosylation site (supplementary Figure 5.S2). The O-linked glycosylation in plants like other eukaryotes takes place in the Golgi apparatus. Since the VHH-IgAs were KDEL tagged for ER retention, no O-linked glycosylation was ideally anticipated. Thus the net putative sites for glycosylation should ideally be just the two asparagine residues bearing N-linked glycosylation. The given possible permutation of glycosylation at these sites, would explain occurrence of three aberrant bands but not 4 bands as- one with no glycosylation, the other with one site being glycosylated while the third high molecular weight band bearing glycans at both asparagine residues. Glycoprotein staining of these 4 bands with

Periodic acid-Schiff's reagent (Kapitany and Zebrowski, 1973), indeed showed that only two of the high molecular weight VHH-IgA bands were glycosylated (Figure 5.11), while the lower two bands (counter stained with Coomassie) did not seem to bear any glycan.

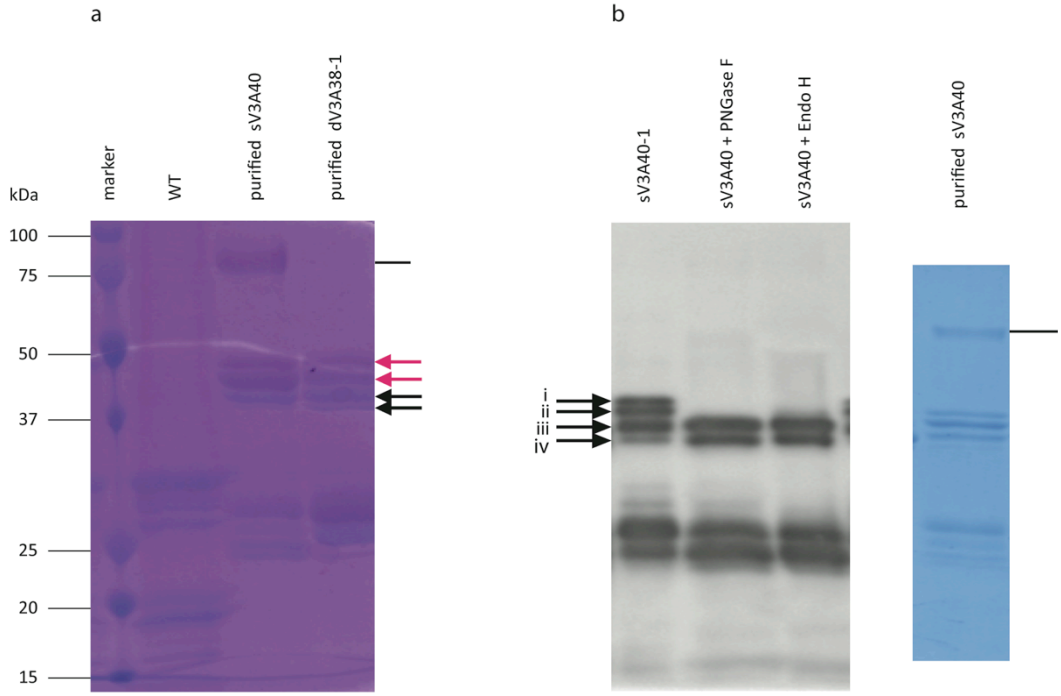


Figure 5.11: Glycosylation analysis of the seed made IgAs. Panel 'a' shows the result of periodic acid Schiff (PAS) stained gel with purified sSIgA and dIgA antibodies from the sSIgA expressing T3 seeds of the line sV3A40-1, and T3 seeds of dIgA expressing line dV3A38-1 (see label on gel) as compare to wild type (WT) seed extract. Of the 4 VHH-IgA bands (~50kDa to ~37kDa), the upper two bands were stained pink with PAS, indicating the presence of glycans (pink arrows), while the lower two bands (counter stained with Coomassie) were unglycosylated (black arrows). The bar indicates the porcine secretory component (~75 KDa) in purified sV3A40-1 seed extract, which is also glycosylated (a). The PAS staining results (a) were affirmed by endoglycosidase analysis with PNGase F and Endo H of the same purified sIgA antibodies from T3 seed extract of sV3A40-1 shown in panel 'b', where the 4 VHH-IgA bands (black arrows labeled i, ii, iii, iv) on cleaving of glycans reduce to two bands (iii, iv). The immunoblot was developed with anti-porcine IgA polyclonal antibody conjugated to HRP. In both the panels 'a' and 'b' the proteins were separated by a 12% SDS-PAGE.

On endoglycosidase digest with PNGase F and Endo H (which excises the N-linked glycans by specifically cleaving of at the beginning of the first GlcNAc or between the first and second GlcNAc, respectively) the high molecular weight bands were reduced to the molecular weight of two lower bands; consequentially the abundance (signal intensity) of the lower two bands increased (Figure 5.11 and supplementary Figure 5.S3). This reconfirms that only the two high molecular weight proteins are glycosylated, and they bear N-

linked glycans. However this only partially explains the 4 different bands. The two lower molecular weight bands with excised N-linked glycans could have different molecular weight perhaps due to truncation of the terminal peptide by proteases. Or the third band (indicated as band iii, in Figure 5.11) still harbors glycans with antennary structures that were inaccessible to PNGase F or Endo H. On comparing the shift in signal intensity as the measure of relative abundance before and after endoglycosidase treatment (Figure 5.11 and supplementary Figure 5.S3), it seems that most of the high molecular weight sugar bearing VHH-IgA is converted to the lowest molecular weight (~37 kDa, band iv, in Figure 5.11), while the accumulation of the third band (band iii, in Figure 5.11) only marginally increases. Further detailed analysis of the glycans with sensitive tools based on mass spectrometry will help in unraveling the post-translational modification in these VHH-IgAs; and further the C-terminal end sequencing will help in exploring the truncation hypothesis. Our preliminary analysis of the nature of the glycans shows that the glycans on the VHH-IgAs are predominantly of the high mannose type (relative abundance= Man7> Man8> Man5) (supplementary Figure 5.S4). All the above glycosylation analysis was done with SSL7 (*Staphylococcus aureus* superantigen-like protein 7) affinity purified IgAs from sV3A40 seeds, in which the 4 bands could be well distinguished, and hence easily analyzed. The glycosidase analysis of V2A and V3A lines seem to suggest that there might be differences in glycosylation correlated with the different VHH fused with porcine IgA, however this would also need to be analyzed by sophisticated tools. The glycosylation of proteins plays an important part in its *in vivo* stability and maintenance of tertiary structure (Loos *et al.*, 2011a); it is likely that the variable glycans on VHH-IgAs might influence the assembly of the sSIgA. Nevertheless, the glycosylation of IgA is necessary for its efficacy and stability against proteases *in vivo* (Corthésy and Spertini, 1999). Unraveling of the glycan structures will help in further optimizing and engineering of glycans, which might lead to attaining higher amount of assembled and functional sSIgA.

Along with glycosylation, the proteolytic cleavage also has consequential effect on the accumulation, assembly and functionality of the antibody. Seed tissue due to its lower protease activity is often suggested as a suitable tissue choice for production of heterologous proteins in plants (Lau and Sun, 2009; Muntz, 1998).

However, specific protease activity is indeed seen in seeds and is also suggested to bear a relation with the glycosylation along with the amino acid sequence (Loos *et al.*, 2011a; Loos *et al.*, 2011b; Van Droogenbroeck *et al.*, 2007). Within the VHH-IgA, we observed substantial amount of proteolytically cleaved VHH-IgA fragments (Figures 5.8, 5.9, 5.10 and 5.11) and the total amount of these cleaved/degraded protein was almost equal to the level of accumulation of the 4 VHH-IgA bands in total (supplementary Figure 5.S3, see area under the curve). From the molecular weight of the fragments determined under reducing conditions it seems that, the cleavage results in removal of about 7-15 kDa mass from the terminal ends (perhaps N- or C-terminal) of the intact VHH-IgA.

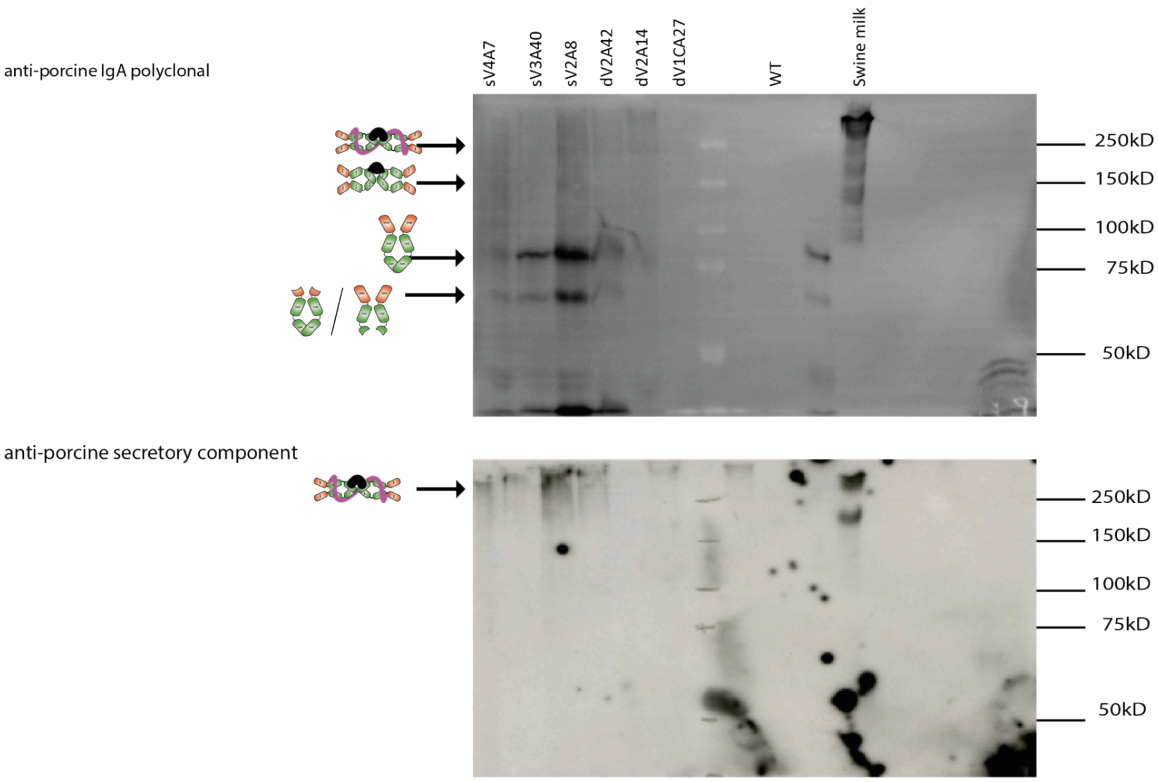


Figure 5.12: Visualisation of complexes under non-reducing condition. The sIgA (sV4A7, sV3A40 and sV2A8) and dIgA (dV2A42, dV2A14 and dV1cA27) seed extracts were separated under non-reducing conditions on a 7% SDS-PAGE and immunoblotted. The same immunoblot developed with polyclonal anti-porcine IgA (top) and with monoclonal anti-porcine SC (bottom) successively after stripping the previous detection antibodies is shown. The dimeric mIgA (80 kDa), the suggested 60 kDa alternative versions of mIgA, the probable dIgA and sSIgA bands are indicated by the cartoon next to the arrow. WT stands for wild type control seed extract while defatted swine milk was used as comparative reference.

Under non-reduced condition, the VHH-IgA dimers of ~80 kDa and another of ~60 kDa were seen (Figure 5.12). The 60 kDa dimer could be the result of union of such proteolytically cleaved fragments (as illustrated in the cartoon besides the gel picture in Figure 5.12). Cleaving of the anterior VHH bearing portion would ideally render the 60 kDa dimer incapable of binding to the antigen, on the contrary excision of the tail end of porcine IgA Fc i.e. the constant α3 domain will not inhibit its antigen binding capacity. In theory, the latter 'Fc tail nibbled' molecule would bind to the antigen with similar strength as the bivalent mIgA, since its bivalency resulting of the disulphide bond within the hinge would be intact. However such a molecule with truncated C-terminal end would not be able to assemble into dIgA or subsequently form sSIgA. We did not evaluate if the ~60 kDa dimer binds to the antigen; evaluation of this should be feasible with a FaeG based immuno-precipitation assay.

In vitro inhibition of the pathogenic F4$^+$ETEC to gut villous enterocytes

The merit of multivalent sSIgAs is its potential to agglutinate and cross-link bacterial pathogens and thus prevent their attachment to the cellular receptor and avoid establishing infection (see immune exclusion Figure 5.1, a) (Ma *et al.*, 1990). The ELISA based screening assured selection of plant lines that produced assembled antigen-binding IgA antibody formats. Further we evaluated the potential of the seed made three IgA variants in inhibiting the live pathogenic F4$^+$ETEC bacterial attachment to its receptor on porcine gut villous enterocytes, *in vitro*. All the IgA antibody formats bearing the 4 different antigen binding VHH (4VHH x three IgA formats) were effective in inhibiting bacterial attachment (Figure 5.13 shows results of sSIgA producing seed extracts). This test being semi quantitative, does not allow the quantitative comparison of efficacy between the mIgA, dIgA and sSIgA. Comparison of mIgA, polymeric IgA and SIgA in previously published literature, states that the only difference between the mIgA and the dIgA format is the enhanced avidity in the latter, due to increased antigen binding valence. While the efficacy of the SIgA and dIgA bearing similar number of antigen binding paratopes, is usually the same. The SC does not add to the net avidity effect of the dIgA, but attributes to the stability of the complex and increases it retention time at the mucosal surfaces (Corthésy, 2003; Strugnell

and Wijburg, 2010). In addition, the glycan of the SC can act as a barricade to pathogens by competing either with the pathogen or the mucosal epithelial cells, for example glycosylated SC can prevent human group A erythrocytes from agglutination by ETEC, while glycosylated human SC competes with *Clostridium difficile* for the toxin A receptor on human epithelial cells (Corthésy, 2002; Dallas and Rolfe, 1998; Giugliano *et al.*, 1995). It would be interesting to investigate if the plant made porcine SC plays a role in sSIgA interaction with ETEC.

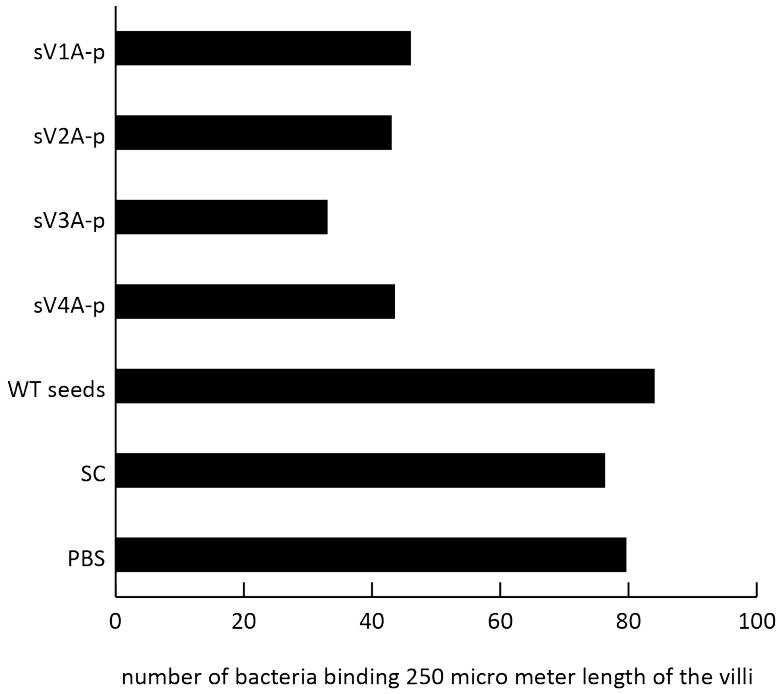
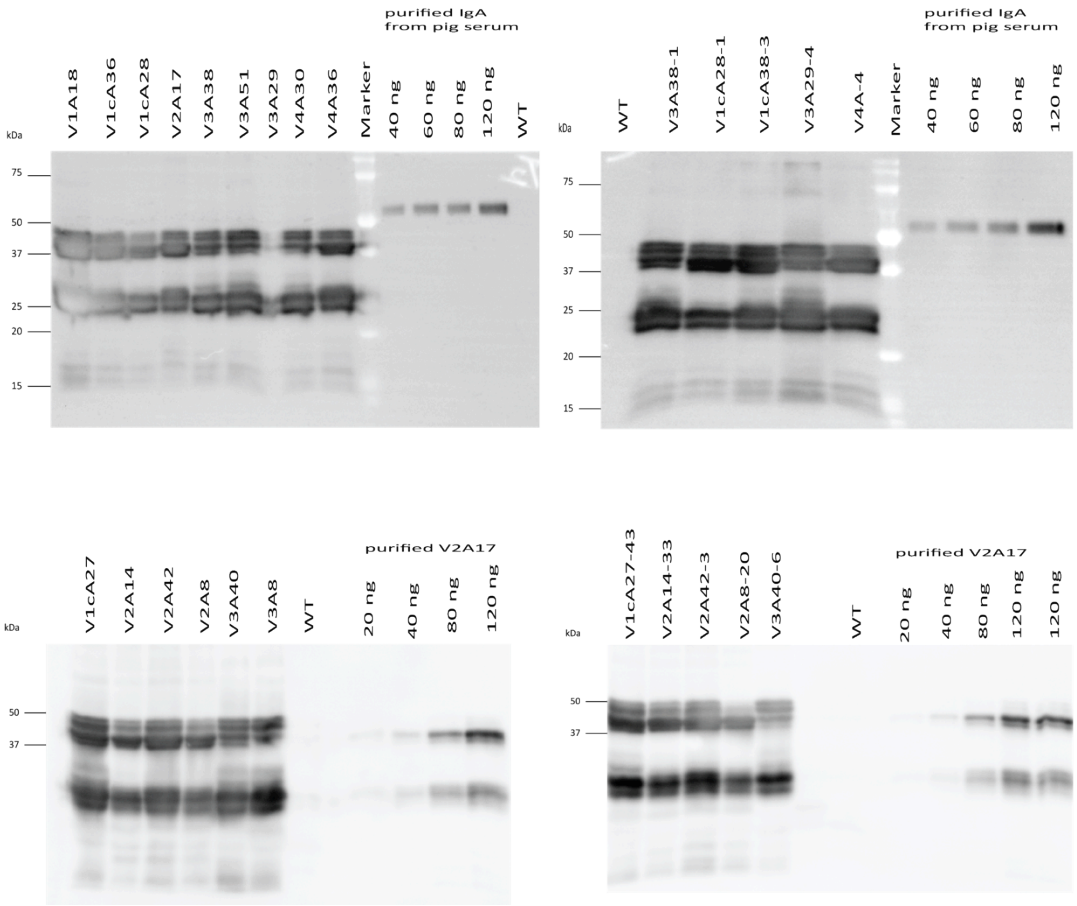


Figure 5.13: Inhibition of bacterial attachment to the porcine gut villous enterocytes *in vitro*. In the semi-quantitative *in vitro* villous binding inhibition test, 100 µl of seed extract made from 4 collective pools (sV1A-p, sV2A-p, sV3A-p, sV4A-p) of T3 seeds of sV1A, sV2A, sV3A and sV4A expressing lines (similar expression level) prevent attachment of F4$^+$ETEC bacterial cells to microvilli. Seed extract from a T2 transformant expressing secretory component alone (Sc) did not inhibit the F4$^+$ETEC attachment, nor did wild type (WT) seed extract; both of which show attachment of bacteria similar to phosphate buffer saline used as negative control. (N.B: All the seed extracts were made by suspending 8 mg of crushed seeds in 200 µl of extraction buffer).

Quantification of antigen binding VHH-IgA chains within the selected high expressing mIgA, dIgA and sSIgA lines

The *in vitro* efficacy results obtained with the anti-F4$^+$ETEC sSIgA antibody formats, paves the way for its future prophylactic application. Prior to which quantification of the functional seed made IgA antibodies it was necessary to formulate a therapeutic prophylactic dose. However the background presented

by the 4 aberrant VHH-IgA bands, the degradation, existence of bi-species and the suggested functional truncates of VHH-IgA Fc— make it difficult to precisely measure the functional units, hence we used a combination of quantifying tools for measuring the functional seed made anti-F4^{+}ETEC IgA.



Transformant plant	VHH-IgA accumulation in T2 seeds (approximate % of seed weight)	VHH-IgA accumulation in homozygous T3 seeds (approximate % of seed weight)
mV1A18	0.5	n.d.
mV1CA28	0.5	0.5
mV1cA 38	0.5	0.5
mV2A17	0.6	n.d.
mV3A 38	0.6	0.7
mV3A 51	0.7	n.d.
mV3A29	n.d.	0.8
mV4A 30	0.6	n.d.
mV4A 36	0.8	n.d.
mV4A 4	n.d.	0.7
dV2A14	0.5	0.7
dV3A8	0.5	n.d.
dV1cA27	0.7	0.9
dv2A42	0.6	0.7
sV2A8	0.5	0.5
sV3A40	0.5	0.5

Figure 5.14: Quantification of accumulation of functional IgA units among the sSIgA, dIgA and mIgA expressing plant. The table shows the expression levels of seed produced VHH-IgA in mIgA, dIgA and sSIgA lines, determined by the sum of the 4 VHH-IgA bands (37 kDa to 50 kDa) from the respective immunoblots. immunoblot 'a' and 'b' – shows the T2 and T3 mIgA lines, while 'c' and 'd' show T2 and T3 sSIgA and dIgA lines respectively. However we estimated that only 50% of this total accumulation is functional i.e. on an average 1% of total soluble protein or 0.2% of seed weight. Each lane contains 8 µg of total soluble protein, and the VHH-IgA bands were detected with polyclonal anti-Pig IgA serum. (n.d. stands for not determined).

As discussed earlier, the basic functional units are the antigen binding VHH domains and multiple-valence adds to its avidity. With this understanding we firstly aimed at visualising the FaeG (antigen) binding units by an immunoprecipitation assay followed by immunoblot. The results suggested that all 4 VHH-IgA bands within the expected molecular weight of 37 kDa to 50 kDa recognised the antigen (Supplementary figure 5.S5) i.e. these 4 aberrant bands in principle were functional. We measured the sum of these 4 VHH-IgA bands expressed in all the IgA format producing lines (sSIgA, dIgA and mIgA) by immunoblot, in comparison to the affinity purified seed made antibody or purified porcine IgA from swine serum as reference standards. Thus evaluated, the average accumulation of mIgA in the T2 seeds was observed to be about 0.5 % of seed weight (Figure 5.14) roughly corresponding to ~2.5% of the total soluble seed protein. Furthermore the accumulation in the homozygous T3 seed stocks raised from these single locus mIgA lines was also measure. Homozygous seed stocks not only facilitates the upscaling, but also the accumulation of heterologous protein expressed in Arabidopsis seeds has been reported to increases in the T3 homozygous seeds perhaps due to doubling of gene dosage (De Jaeger *et al.*, 2002; Van Droogenbroeck *et al.*, 2007). Such increase in T3 homozygous line producing VHH-IgA was seen only in one line by a marginal increase of 0.12% of seed weight, where as the overall average in the T3 stock was about 0.6 ± 0.1% of seed weight (N.B. 20% of Arabidopsis seed weight is protein). There were also instances where the accumulation barely increased from T2 to T3 homozygous seeds (mV1cA38, in Figure 5.14).

The accumulation of the VHH-IgA in the sSIgA and the dIgA lines was also comparatively similar to the expression measured in the monomeric VHH-IgA lines with an average of 0.5% accumulation in T2 seeds and an increase in some T3 homozygous seed stock to an average of 0.7% or 0.8% of seed weight (Figure 5.14). Thus seemingly, the co-expression of the J chain or the SC did not have any significant enhancing effect on accumulation of the VHH-IgA of V1A, V2A, V3A, or V4A as reported by Ma *et al*. in tobacco produced SIgA (Ma *et al.*, 1995).

ELISA-based quantification of each of these functional units is theoretically possible but requires that each of the respective IgA format be purified, to serve

as standard reference; as the affinity of each of these formats with different VHH at their antigen-binding domain is different. With a few purified mIgA standards we evaluated via ELISA that only half of the measured sum of the 4 VHH-IgA bands is functional. Form our calculations based on ELISA and immunoblot data we estimate that the overall functional IgA in the oligomeric pool (sSIgA, dIgA and mIgA) is about 1% of the total soluble protein, which is 0.2% of the seed weight.

Conclusion

In conclusion, this study shows for the first time that a simplified version of secretory IgA (sSIgA) can be produced effectively in plants, which is a viable expression platform for recombinant secretory IgA production (Paul and Ma, 2011). In comparison to the previously produced and now commercialised CaroRx® antibody in stably transformed tobacco lines by successive filial crossing (Ma *et al.*, 1995), our co-transformation method is fast. Further both, our 2PM-PCR and ELISA-based approach enable screening of vast amounts of primary transformants for identifying the best expressing lines. The cloning approach utilised, incorporating database sequestration provides an example that synthesis of customised recombinant sSIgAs can be attempted for different species, in which the sSIgA is yet uncharacterised. The in seed production, together with prospects of oral in feed delivery would makes sSIgAs particular worth evaluating for oral mucosal passive immunisation against enteric veterinary diseases (we evaluate this possibility in Chapter 6). Swapping of the VHH against other porcine enteric pathogens can be done conveniently in the current anti-F4-VHH-IgA construct. Once high expressing primary transformants for the new VHH-IgA are identified, it could be crossed with the porcine SC and J chain expressing lines (porcine SC + J chain expressing T2 plants, Figure 5.7) from our co-transformation library, for future convenience of producing porcinised- sSIgA or dIgA.

Along with these merits our results show that there is a window of opportunity to further develop and optimise the seed production platform for maximum production of sSIgA. Our current estimates is that only about 50% of the VHH-IgAs expressed are functional, and of the 50% the ones that form sSIgAs and

dIgAs in seeds are a further fraction. The assembly of the sSIgA and dIgA in seeds most likely depends on the molar accumulation and stoichiometry of SC and J chain, where the glycosylation might have a role to play. Crossing the high accumulating mIgA, dIgA, sSIgA homozygous lines with glycosylation mutants and ones with modified human-like glycosylation pattern will help in further unravelling the potential of glycosylation in assembly of sSIgA. Also manipulations within the regulatory elements can help with controlling of the molar expression of each element, thus enabling optimum stream line production and avoiding wastage of unassembled elements.

Amongst these merits and demerits, perhaps the most interesting piece of evidence is that all the formats of anti-F4$^+$ETEC sSIgAs are functional in inhibiting the bacterial attachment to the gut villous enterocytes *in vitro*, and in Chapter 6 we unravel the *in vivo* efficacy of this simplified secretory IgA (sSIgA).

Experimental procedures:

Fusion of the VHH to Pig Fc

The IgA^b was chosen as the fusion partner for production of VHH-IgA based antibodies. The entry clones were made as described for VHH-IgG fusion in Chapter 4. Briefly, the codon usage within the native sequence of IgA^b (derived from UniProt accession no. U12594) was compared to that of Arabidopsis seed storage proteins and was manual optimised. The first V1A fusion was chemically synthesised within the attB1-attB2 Gateway recombination sites in the following 5' to 3' direction— 'EcoRI restriction site, Kozak sequence (CCACC), 2S2 seed storage signal peptide sequence, VHH V1fused with the codon optimised sequence of IgA^b, a KDEL endoplasmic retention signal, stop codon and BamHI restriction site'. In a Gateway BP reaction, this stretch of DNA was recombined with the Gateway Donor plasmid pDon221 (bearing kanamycin resistance) according to manufacturers instruction, which results in an entry clone, which was named pEV1A.

For cloning of other three anti-$F4^+$ETEC VHHs– V2, V3 and V4 in fusion with IgA Fc the DNA sequence for restriction site for EcoRI + Kozak sequence + 2S2 seed storage signal peptide + each of the three VHHs was chemically synthesised. Then Using the EcoRI and the BstEII (located in framework4 of all VHHs) restriction sites, the VHHs V2, V3 and V4 with the 2S2 signal peptide were swapped with the V1 from entry clone pEV1A. Thus, Entry clones (E) pEV2A, pEV3A and pEV4A were made.

Porcine J chain and secretory component

The amino acid sequence of the porcine J chain is not defined, however from homology based search as compare to the human J chain (accession no: NP_653247) within the porcine EST database we could identify the J chain homologous gene from the cDNA library made from alveoloar macrophages (accession no.: AK231006). This gene was then defined as porcine J chain. Using the signal peptide predictor (SignalP 3.0) the signal peptide of J chain was identified (Bendtsen *et al.*, 2004) and replaced with the signal peptide of 2S2 Arabidopsis seed storage protein. Thereafter, using parameters as define earlier

(Chapter 4) the codon usage was optimised for Arabidopsis seed. The coding sequence for the endoplasmic reticulum retention signal (KDEL) was added on the 3' end (C-terminal end) followed by a stop codon, this DNA was chemically synthesised within the attB1 and attB2 Gateway sites, and the Kozak sequence (nucleotides- CCACC) was added upstream of the signal peptide during this process. Further via Gateway BP recombination reaction this construct was recombined into the pDONR221 (kanamycin resistance) (according to Gateway manual) to result into an entry vector, which was called pEJ.

Secretary component (SC) is the resultant product of proteolytic cleavage of the polymeric immunoglobulin receptor (pIgR), which results in either free SC or associated with the dimeric IgA. The sequence of the porcine SC was derived from the published sequence of porcine pIgR (Kumura *et al.*, 2000)(accession no.: NM_214159, UniProt accession no. Q9N2H7). The endogenous signal peptide of pIgR was determined using prediction tool (Bendtsen *et al.*, 2004) and then replaced by the signal peptide of the Arabidopsis 2S2 seed storage proteins. After the signal peptide the first 579 aa length spanning until the 5th domain (corresponds to the 585 aa of human SC) was selected and defined as porcine SC. The nucleotide sequence of this porcine SC bearing Arabidopsis 2S2 signal peptide was also codon optimised as described above, and chemically synthesised with attB1 and attB2 flanking sequences. This construction was cloned in the multiple cloning site of pUC57, and then later recombined in a BP reaction with the pDONR221 to get the entry plasmid pESC with kanamycin resistance (in accordance to the Gateway manual).

Gateway recombination into destination vector and *Agrobacterium* mediated transformation

All the entry clones except the pEJ were recombined via an Gateway LR reaction into the destination vector pPhasGW (Morandini *et al.*, 2011) following the Gateway instruction manual (as described in Chapter 4). The T-DNA of which bears the *nptII* gene, which confers for kanamycin resistance in plants. The 4 expression clones (X) bearing the VHH-IgA fusion were named: pXV1A, pXV2A, pXV3A, pXV4A and the expression plasmids bearing SC was called pXSC.

To facilitate the identification of co-transformed plants, the entry plasmid pEJ was recombined into a multisite gateway cassette pBm43GW,0 the T-DNA of which bears the *bar* gene that confers for phosphinothricin (PPT) selection (Karimi *et al.*, 2005). According to Multisite Gateway instructions, 3 entry plasmids- pEJ (bearing J chain, attL1-L2), pEPhas (bearing Phaseolin promoter, flanked by attL4-attR1 (De Wilde, 2012)) and pEArc600 (3' regulatory sequences bearing arcelin terminator, flanked by attR2-attL3 (De Wilde, 2012)) were recombined to result in the expression plasmid which was called pMXJ.

The expression plasmids were checked by restriction digestion sequencing of the gene of interest and flanking regions. On confirmation, the plasmids were transformed into Agrobacterium strain C58C1Rif^{R} and used for floral dip transformation as described previously in Chapter 4. With the slight modification, To produce plants expressing a complete sSIgA complex (with the VHH-IgA fusion, the J chain and the SC chain) three *Agrobacterium* strains carrying the plasmids pMXJ, pXSC and one of the plasmids pXV1A, pXV2A, pXV3A or pXV4A; each were inoculated in 20ml YEB medium without any antibiotics overnight and when the OD_{600} reached about 1.7; 3.3 ml of each of the three cultures were mixed together. This mixture was made up to 50 ml with dipping solution (10% sucrose and 0.05% Silwet solution in water) and used for the floral dip transformation. For each of the 4 different VHH-IgA fusions (the 4 mixes: V1A+SC+J; V2A+SC+J; V3A+SC+J; V4A+SC+J) ten *Arabidopsis thaliana* (Col 0) plants were transformed to produce different formats of IgA antibodies bearing the 4 different antigen binding domain.

From each transformed plant 1000 seeds were sown on kanamycin bearing MS medium and the primary transformant were selected. (for detailed protocol of sowing, selection of primary transformants and plant growth conditions see Chapter 4).

Callus Induction for selection of J chain positive T1 transformants from co-transformed sSIgA lines

The callus induction test was used to determine the presence of the J chain bearing T-DNA within the transformants screened positive on kanamycin selection plates (SC and/or VHH-IgA present). The plantlets on the selection

plate were numbered and one leaf per plant was taken for the callus induction test. The plates were opened in a laminar flow and a representative leaf from each numbered plant, was cut transversely through the midrib with a sterile sharp blade, this leaf piece per plant was transferred to a Petri plate with grid markings, containing callus induction medium (4.308 g/l Murashige and Skoog salts, 0.5 g/l MES, 30 g/l sucrose, pH 5.7, with 0.7% agar) with 100 mg/ml PPT, MS Vitamins, 0.1 mg/l NAA (1-Naphthaleneacetic acid) (auxin) and 1 mg/l BAP (6-Benzylaminopurine) (cytokine). One leaf piece was placed per square in the pre marked grid of the Petri plates with its abaxial surface touching the medium. Each leaf was numbered similar to its parent plant. The lids of the Petri dishes were sealed with porous adhesive tapes (Milipore) and incubated in the growth chambers at 21°C with 16 hours light and 8 hour dark photoperiod (light intensity 80 $\mu E/m^2/s$). At the end of three weeks, growth of healthy callus was indicative positive for bearing J chain T-DNA. Thus perspective dimeric (4xVHH-IgA + J chain) and sIgA (4xVHH-IgA + J chain + SC) producing lines could be segregated from pool of kanamycin resistant T1 transformants (VHH-IgA and/or SC).

PCR based screening of the SC, J chain and VHH-IgA genes in co-transformed plants

Cetyltrimethylammoniumbromid (CTAB) based high through put DNA extraction

The DNA was extracted from green siliques using cetyltrimethylammonium bromide (CTAB) method modified for high-throughput extraction in 96-tube micro-rack format. Each tubes was filled with 2 steel balls of 3 mm diameter each, into which two siliques were harvested. Once filled the entire rack was snap chilled in liquid nitrogen and the frozen siliques were crushed to powder using the mixer mill (MM400-Retsch) at 30 Hz frequency of oscillation for 30 seconds. The rack was given a brief spin (eppendorf centrifuge -5810 R equipped with adapters for 96 tube multi racks) and then 400 µl of extraction buffer (2 x CTAB buffer: 2% w/v CTAB, 100 mM Tis-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl and 1% w/v polyvinylpyrrolidone) was added and vortexed to homogenise the crushed material. This mixture was incubated at 37°C for 45 minutes, cooled down to room temperature, 400 µl of chloroform was added mixed by brief

vortexing and centrifuged for 15 minutes at 3000g. Post centrifugation, 300 µl of upper aqueous phase was transferred to tubes (in 96 multi-rack) pre dispensed with 400 µl of isopropanol, mixed well and incubated at room temperature for 5 minutes. The mixture was centrifuged for 45 minutes at 3031g and the supernatant was discarded by decanting the rack. To these tubes 200 µl of 70% ethanol was added and centrifuged for 15 minutes at 4°C and 3000g. After this ethanol wash, the residual ethanol was poured off and the pellets were air dried at 37°C for 30-45 minutes. To the dried DNA pellet 200 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) was added and left at 37°C for 30 – 45 minutes; 1 µl of this dissolved DNA was used per polymerase chain reaction (PCR).

2-primer Multiplex PCR (2PM-PCR)

One microlitre of extracted plant DNA was added to the 2PM-PCR mix containing 1.5 mM $MgCl_2$, 0.2 mM dNTP's mix, 0.2 mM of forward primer - EK2S2 and reverse primers – Sybuc 93 (sequence below), 4.5 units of Taq polymerase (Invitrogen) and 5 µl of Taq polymerase buffer in 50 µl final reaction volume. Ninety-six samples were processed simultaneously in PCR plate. The PCR plate was incubated in a thermocycler (Biorad, iCycler). After 5 minutes of initial denaturation at 95°C, 30 amplification cycles were carried out, each cycle with 45 second of denaturation at 95°C, 45 second of annealing at 57°C, followed by 45 seconds of elongation at 72°C. On completion of 30 cycles the reaction was incubated at 72°C for 5 minutes as final elongation step. After the completion of the reaction, the PCR products were analysed by DNA agarose gel electrophoresis. To the reaction product 5 µl of loading dye (containing Orange G) was added and 10 µl of this mix was loaded into the wells of 1% agarose gel in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. The bands were separated by applying 100 volts of potential difference for 1 hour. The agarose gel was stained with ethidium bromide for 5 minutes and then visualised under UV light and documented (electrophoresis and documentation system 120, Kodak with Kodak 1D software version 3.1).

The chosen pair of primers has complementary sites within the common elements (sense strand primer in the signal peptide, while for anti-sense primer in the terminator) flanking the gene of interest (SC, J, and VHH-IgA). Thus using a

single PCR, the presence of each of these genes could be detected, as each gave rise to an differential size amplicon (SC: 1832 bp, VHH-IgA: 1178 bp, and J chain: 503 bp).

Sense strand primer- EK2S2:
GGAATTCCCACCATGGCAAACAAGCTTTTCCTCGTCTGCGCAACTTTCGCCCTC

Anti-sense primer- Sybuc 93: ACAGGGAAGGTGGTTTTGGG

Screening for transformants expressing high amount of functional antibodies:

The protein was extracted from the seeds using the high throughput extraction procedure, the protein concentration was measured, and the concentration of TSP was adjusted to 1 mg/ml as described in Chapter 4. Based on the FaeG immobilised ELISA set up the seed extracts were analysed for seeds stocks expressing highest amount of functional antibody as described in Chapter 4, with the exception that, for detection of VHH-IgA antibodies, 100 µl of the polyclonal anti-porcine IgA antibody raised in goat conjugated to HRP (AbD serotech AA140P) diluted 1/10,000 in 2% skimmed milk was used.

In order to determine the presence of functional dimeric IgA [4 x (VHH-IgA) + J chain] and sSIgA antibodies via ELISA, all the steps until seed extract addition to FaeG immobilised ELISA wells was followed (Chapter 4). To detect dimeric IgA antibody, the anti-J chain monoclonal antibody (Thermo scientific MA1-80527) was used, diluted 1/1000 in 2% skimmed milk in PBS. Whereas, to detect the secretory sSIgA antibody [4 x (VHH-IgA) + J chain + secretory component] the monoclonal anti-pig secretory component antibody (Thermo Scientific MA1-80544) was used. The primary antibody was incubated for 90 minutes followed by 5 washes (0.1% Tween20 in PBS). The secondary antibody, anti-mouse polyclonal made in sheep conjugated to HRP (Amersham Bioscience, NA931V) was used, diluted 1/5000 in 2% skimmed milk in PBS, the plate was incubated at room temperature for 1 hour. Thereafter, the plate was washed and 100 µl of the substrate TMB was added into each well. After incubation for 30 minutes blue colour developed, which was read at 640 nm, using the VERSAmax tunable microplate reader (Molecular Devices, USA). From the optical density measured

at 640 nm the plants were ranked according to the expression level for dimeric or secretory IgA. These plant extracts were then analysed using SDS PAGE and immunoblot techniques as described in Chapter 4 and (De Buck *et al.*, 2011).

Polyclonal anti-porcine IgA antibody raised in goat (AbD serotech AA140P) diluted 1/4000 in 2% skimmed milk were for detection of VHH-IgA chains. In case of J chain detection, the primary antibody, anti-J chain monoclonal antibody (Thermo scientific MA1-80527) diluted 1/4000 was used; while for SC detection monoclonal anti-pig secretory component antibody (Thermo scientific MA1-80544) was used diluted 1/2000. The secondary anti-mouse polyclonal made in sheep conjugated to HRP (Amersham Bioscience, NA931V) was always used diluted 1/4000 in 2% skimmed milk.

The commassie stained gels and immunoblots were documented using the Chemidoc™ system (Biorad); and using the Image Lab software the protein bands were quantified using affinity purified V2A antibody (from V2A17 T2 seeds) or purified IgA from swine milk as reference.

Determination of single locus

For VHH-IgA producing plants, the highest expressing lines were selected and 64 surface sterilised seeds from each of these lines were sowed on MS plates with kanamycin. In case of the dimeric IgA and sIgA, the plants with the highest OD values in functional ELISA correlating to the highest accumulation of assembled molecules were chosen for the single locus determination. Approximately 192 T2 seeds from each of the line were vapour sterilised, 64 seeds of these were sowed on K1 media with kanamycin selection, 64 on phosphinothricin and 64 on plated with dual selection of phosphinothricin and kanamycin.

The plates with seeds were sealed with porous tape (Millipore), kept in dark at 4°C for 48 hours for stratification and transferred to growth room at 21°C with 16 hours light and 8 hour dark photoperiod. After 2-3 weeks the resistant plants could be well distinguished from the sensitive ones. These numbers were compared via chi square test to determine the lines with significant 3:1, resistant : sensitive segregation pattern, which is indicative of single locus insertion (De Neve *et al.*, 1997). From the single locus plants the homozygous T3 lines were

identified (see appendix for detailed segregation tables).

Affinity purification of seed produced antibodies

For purification of VHH-IgA and all IgA antibody formats the *Staphylococcus aureus* superantigen-like protein 7 (SSL7)/Agarose resin (InvivoGen) was used, either 5 cm long columns were hand-packed then plugged into AKTA system or the proteins were purified via batch system, depending upon the quantity of purified antibodies desired. In principle the resin was washed and then equilibrated with the binding buffer pH 8 and then the protein extract was applied to it, the unbound proteins were washed away with PBS. The bound antibodies were eluted by changing the pH with 0.1 M glycine buffer pH 3. Immediately after, the column and the eluted fraction was neutralised with 1 M Tris pH 7.2. The resin was stored in 20% ethanol in PBS.

Piglet villi binding inhibition test

The villi binding inhibition test was performed in accordance to Coddens *et al.*, 2009 (also see Chapter 4).

Glycan analysis by mobility shift assay on glycosidase treatment

To determine the nature of N-linked glycans present on the recombinant protein, the N-linked glycosidase Endo H and or PNGase F were used according to the manufacturer's instructions. In short, the VHH-IgG and VHH-IgA antibodies were first denatured by heating at 100°C for 10 minutes in a denaturing buffer containing 5% SDS and 0.4 M dithiothreitol (DTT). Then the glycosidase enzyme was added and incubated at 37°C. After about 60 minutes of incubation, the reaction was stopped by adding Laemmli buffer and heating the sample at 98°C for 8 minutes. This sample was then loaded on to a SDS-PAGE gel and immunoblotted to determine the shift in the mobility of the protein after removal of N-linked glycans.

The Periodic acid Schiff's staining of the glycoproteins was performed using the Glycoprotein staining kit (Thermo Scientific) according to manufacturer's instructions.

Supplementary data:

MTRFFYACLL AIFPVVSMKS PIFGPQDVSS VEGSSVSIRC YYPATSVNRH [50]
SRKYWCRIGA KGRCTTLISS EGYISKDYKG RA**N**LTNFPE**N** GTFVMDIGHL [100]
TRGDSGLYKC GLGISSRGLS FDVSLEVSQG PGQIGDVHVY TADLGSTVTI [150]
NCPFKSENAQ KPKSVYKKLG QIRVLVIDSN GYLNN**N**FTNR AHLSIQGTNQ [200]
LVFSFVINRI QLRDAGIYIC QAGDEESSAD LQVLKPEPEL IYGDLRGSVT [250]
FDCALGQEMA NVAKFLCQLK NGKTCNVVIN TLGKKAQDFE GRILLTPKEN [300]
SHFSVHITGL RKEDAGHYLC GAHPDGEPKE GWPVQAWQLF INEDTMIPPR [350]
SSVVRGVVGG SVAVTCPYNP KETNSLKYWC RWEENENGRC PQLVESSGLV [400]
NEQYEGRLAL YEEPG**N**GTFT VILNQLTNRD AGFYWCLTNE DSRWRSTVEL [450]
KIVEGEPNLK LPE**N**VTAWVG ETLKLSCHFP CKFYSYQKYW CKWSNTGCRA [500]
LPSQDEGQSQ AFVNCDKKSQ IISLNLNPVR KEDEGWYWCG VKDGLHYGET [550]
GAVYVAVEQK AKGSGDARLA NAAPAPAEDA IEPRARETEN EVLLDPSLIA [600]
EDRAVKDGEG PASGSGVPAA PGSSVGQGGG SKAVVSTLVP LALVMLVGAV [650]
TIGVLRARHR KNVDRISIRS YRTDISMSDF ESSRDFGAHD NAGATLDTQE [700]
TSLGGKDRFT TTTENTMEDK EPKRAKRSSK EEADKAFTTF LLQANSIIAA [750]
TQNGPREA [758]

Supplementary figure 5.S1: Amino acid sequence of the porcine polymeric immunoglobulin receptor. The sequence of the porcine SC, spanning the 5 extracellular domains is underlined, the native signal peptide is bold face, the predicted N-Link glycosylation sites within the extracellular domain are bold faces in red and the amino acid count per line is indicated in square brackets at the end of the line.

QVQLQESGGG LVQPGGSLRL SCTASGSISS INAMGWYRQA PGSKREFVAH

ITNTGVTEFA DSVKGRFTIS RDNAKTTVDL QMNSLKPEDT AVYYCAATDW

GTLLIKGIDH WGKGTQVTVS SDPCPQCCKP SLSLQPPALA DLLLGS**N**ASL

TCTLSGLKKS EGVSFTWQPS GGKDAVQASP TRDSCGCYSV SSILPGCADP

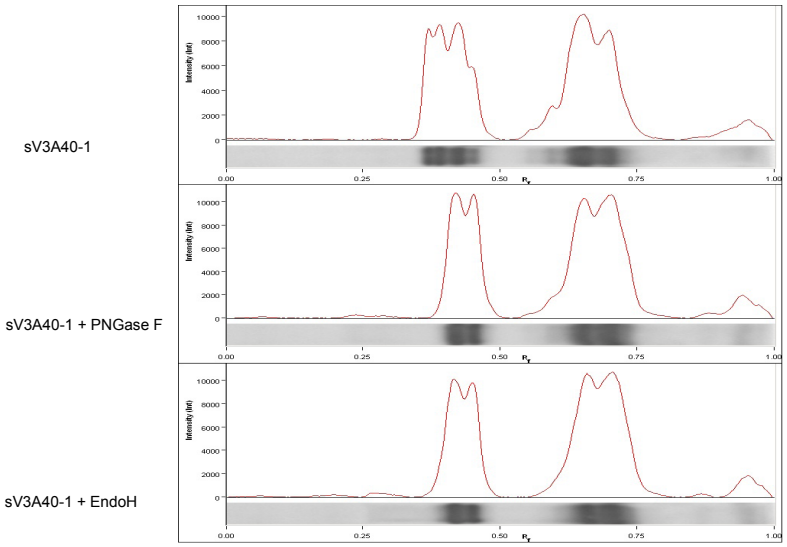
WNKGETFSCT AAHSELKSAL TATITKPKVN TFRPQVHLLP PPSEELALNE

LVTLTCLVRG FSPKDVLVRW LQGGQELPRD KYLVWESLPE PGQAIPTYAV

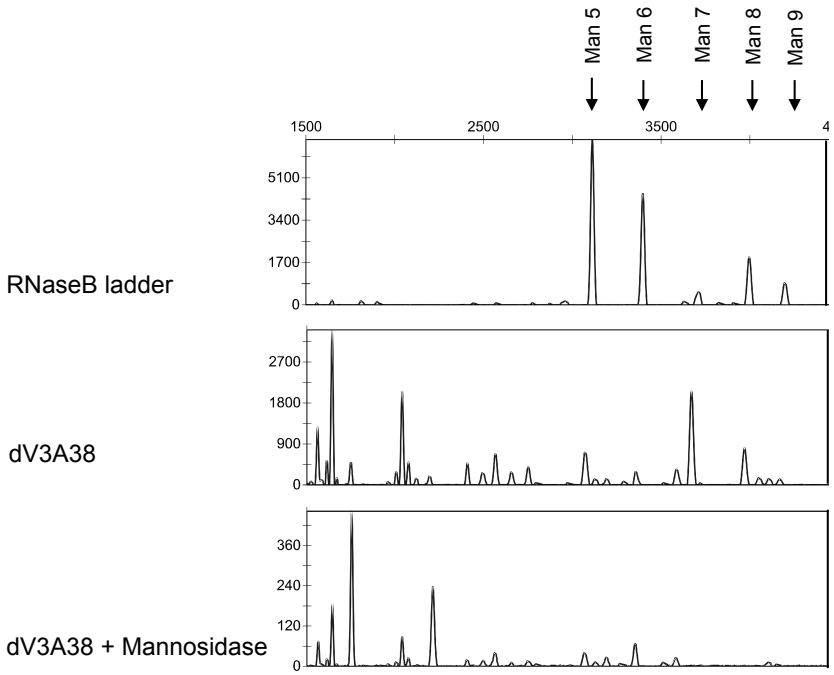
TSVLRVDAED WKQGDTFSCM VGHEALPLAF TQKTIDRLAG KPTHV**N**VSVV

MAEAEGICY**K DEL**

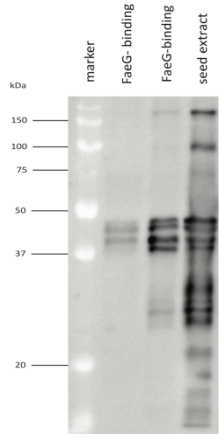
Supplementary figure 5.S2: Amino acid sequence of the V2A as representative of the anti-F4 fimbriae VHH fusion to Fc fragment of the porcine IgA^{b}. The sequence of VHH V2 is marked in red, the Fc of IgA^{b} is in black, where the hinge is underlined and the predicted N-link glycosylation sites are bold faced, while the predicted O-linked glycosylation site is indicated with an arrow head. The endoplasmic reticulum retention tag 'KDEL' is indicated in blue (Each line has 50 amino acids in 5 blocks);



Supplementary Figure 5.S3: Alternative representation of the mobility shift assay, where the intensity of the signal of each band is plotted on the graph above it, and shows the shift in the intensity of the signal from first and second glycosylated band (i and ii) predominantly to the 4th band (iv). The total amount of degradation is roughly equivalent to the total accumulation of the 4 bands of VHH-IgA (area under the curve).



Supplementary Figure 5.S4: Analysis of the glycans on the VHH-IgAs reveals high mannose residues. Using the CE-LIF (Capillary electrophoresis – laser induced florescence) method described for determination of plant N-linked glycans; the VHH-IgA bands from the plant line dV3A38 were analysed (Nagels *et al.*, 2011). The preliminary results indicate that the sugars are of the high mannose type (mostly Man 7) (compare middle panel with RNaseB reference ladder on top). On mannosidase treatment these glycans were cleaved off (lower panel). (The glycan analysis was performed by Francis Santens and Bieke Nagels at the unit for Medical Biotechnology headed by Prof. Nico Callewaert, Department for Molecular Biomedical Research)



Supplementary figure 5.S5: FaeG binding VHH-IgA fragments.
The antigen FaeGac covalently linked to agarose beads was used to determine the antibody fragments that bind the antigen, it was seen for several lines that all of the 4 VHH bands between the molecule weights of 37 and 50 kDa detected by anti-pig IgA antibody bind to the antigen FaeG. Representative results from two plant lines have been shown (labelled FaeG-binding) in comparison to the VHH-IgA expressing seed extract (labelled seed extract).

Chapter 6

In vivo evaluation of protection by in feed administered, seed made antibodies against F4-positive enterotoxigenic *Escherichia coli* challenge

Oral passive immunisation - exploring a possibility

Vikram Virdi, Annelies Coddens, Sam Millet, Sylvie De Buck, Bruno Goddeeris, Henri De Greve, Eric Cox and Ann Depicker

V.V. performed the whey competition assay, produced and up scaled the transgenic seeds, and prepared all the feed premix. Further with the help of technical support personnel, under the mentorship of A.C, V.V screened the piglets, standardised the piglet feed-challenge model, performed the piglet-challenge experiment, and process the faecal and blood samples. The results were analysed by V.V and A.C. Statistical analysis were performed by S.M. The receptor phenotypic status analysis was done by A.C, by a villi-binding test. The palatability experiment was performed by S.M. he also formulated the feed and prepared the treatment feed. The animal experiment was designed chiefly by E.C, B.G and A.C in consultation with H.D.G and A.D. The project was coordinated by V.V, and he wrote this chapter. A.C, A.D, H.D.G, S.M and S.D.B edited the manuscript.

Abstract:

F4 fimbriae bearing enterotoxigenic *Escherichia coli* ($F4^+$ETEC) caused post-weaning diarrhoea is one of the most important causes of heavy economic losses to the global porcine rearing industry. With the ban on traditional prophylactic use of antibiotics in several countries, there is a desperate need for alternative prophylaxis against $F4^+$ETEC infection. Of the several alternatives, oral passive immunisation with anti-$F4^+$ETEC antibodies has been by far the most promising. So far the anti-$F4^+$ETEC antibodies were sourced either from animal plasma proteins or from immunised hen eggs. The issues of safety and consistency of anti-$F4^+$ETEC from pig blood and the high cost of producing antibodies in eggs has limited their widespread use.

As a solution, we previously developed anti-$F4^+$ETEC antibodies in the seeds of the model plant *Arabidopsis thaliana* to evaluate a cost effective alternative for specific anti-$F4^+$ETEC oral prophylaxis. These seed produced anti-$F4^+$ETEC antibodies were functional in preventing $F4^+$ETEC bacteria attachment to gut villous enterocytes, *in vitro.* In this study we evaluate the *in vivo* efficacy of these seed produced antibodies to protect against $F4^+$ETEC challenge on oral in-feed antibody administration.

These seed produced antibodies were custom designed for in-feed oral passive immunisation. This customisation was achieved by using the antigen binding 'variable domains of heavy chain only antibodies' (VHH) from lama, which are known to be robust molecules. VHHs selected against the $F4^+$ETEC from lama immune library were grafted on to porcine Fc and expressed in seeds. By fusion to porcine IgG3 Fc and IgA^b Fc, two basic formats i.e. VHH-IgG and VHH-IgA were produced. Further by co-expression of porcine J chain and secretory component dimeric and secretory IgA like antibodies were made from the VHH-IgA. Overall 4 plant lines expressing different VHH-IgG and 12 lines expressing different formats of VHH-IgA antibody were scaled up, and the respective seeds were pooled to form two oligomeric cocktails of each– VHH-IgG and VHH-IgA.

From the two oligomeric pools, three prophylactic feeds were formulated namely: VHH-IgA-20, VHH-IgG-20, VHH-IgG-80 such that a daily intake of 300

grams of feed per pig would administer 20 mg of oligomeric cocktail of VHH-IgA or 20 mg of VHH-IgG or 80 mg of VHH-IgG antibodies, respectively. The efficacy of these 3 feeds was evaluated together with negative control feed containing wild type Arabidopsis seeds, on challenge with pathogenic F4$^+$ETEC.

Shedding of bacteria was seen in piglets of all the 4 groups, however in case of VHH-IgA-20 group the shedding declined progressively every day and reduced to below detection levels by day 4. VHH-IgG-20 group also had swift decline in bacterial shedding, however it was detected post-mortem that 2 of the 3 piglets in this group were insensitive to F4$^+$ETEC since they had low F4 receptors (F4R) hence the protection of VHH-IgG-20 feed would have to be re-evaluated in a prospective study. While within the piglets of the VHH-IgG-80 feed group shedding of challenge bacteria until the end of the experiment till day 11 was observed, and thus VHH-IgG-80 failed to protect. The seroconversion also corroborated the shedding profile; piglets of the VHH-IgA-20 had a lower rate of seroconversion than the negative control or the VHH-IgG-80 group.

The effect of VHH-IgG-20, and the reason for ineffective VHH-IgG-80 needs to be further investigated. While the VHH-IgA-20 seems as a promising prophylactic solution to the F4$^+$ETEC caused PWD. In future, it would be interesting to produce VHH-IgA antibodies in crop seeds, that can be conveniently scaled up to evaluate prophylaxis in a piglet field trial.

Introduction

One of the most important causes of economic loss to the global porcine rearing industry is post-weaning diarrhoea (PWD) (Amezcua *et al.*, 2002; Hong *et al.*, 2006). PWD in piglets is regarded as a multifactorial disease, caused by the overwhelming conglomeration of physical, physiological and psychological stress at weaning that makes the piglets vulnerable to pathogens like F4-positive enterotoxigenic *E. coli* (F4$^+$ETEC) (Madec *et al.*, 2000). The protective immunity provided by maternal milk antibodies during suckling period is discontinued at weaning (Riising *et al.*, 2005; Wilson and Svendsen, 1971), and as a consequence in addition to the looming stress, piglets succumb to infections caused by F4$^+$ETEC. In theory, this protection can be prolonged by oral administration of

antibodies after weaning (Marquardt *et al.*, 1999; Niewold *et al.*, 2007; Yokoyama *et al.*, 1992). Opportunity of oral delivery in feed is particularly interesting for large-scale porcine rearing industry. We envisage that oral delivery of in-feed produced passive immunity during the critical stress window period for about a week or 2 post-weaning may prevent F4$^+$ETEC infections.

A few years ago Harmsen *et al*. (2005) isolated novel VHH against F4$^+$ETEC bacteria for oral passive immunization with the understanding that VHHs are robust antibody fragments. In spite of the group's promising *in vitro* results the VHH passive immunotherapy failed to offer any protection *in vivo*. They reasoned that failure to protect *in vivo* could be due to proteolysis of VHH in the gastrointestinal tract among other reasons (Harmsen *et al.*, 2005). Evidently, oral passive immunization though is an apt route for attaining passive protection at the mucosal surface, it is only guaranteed by antibodies that can withstand the transit through the harsh gastric environment and be functional at the site of infection which for F4$^+$ETEC is the small intestine (Reilly *et al.*, 1997).

Oral in feed delivery of in seed produced antibodies, which are retained within the endomembrane system, in theory enable protective bio-encapsulation (Khan *et al.*, 2012). However their release from the feed matrix, in the elementary canal and functionality there after needs to be experimentally evaluated. With this rationale, we expressed anti-F4$^+$ETEC VHH-IgG Fc and VHH-IgA Fc fusion antibodies in seeds of the model plant– *Arabidopsis thaliana*. Aiming to produce robust antibodies with capacity to withstand gastric denaturation we designed novel chimeric 'porcinised-lama' antibodies. From an immunised lama library, four F4 fimbriae binding variable domains of heavy chain only antibodies (VHH) were carefully selected and grafted on to porcine Fc region (fragment crystallisable region) of IgG3 or IgA^b chains. In seed expression of the VHH-IgG Fc fusion led to production of 4 divalent antibodies, namely: V1G, V2G, V3G and V4G. Building further on the similar strategy, by VHH-IgA fusion we produced 4 divalent IgA like antibodies (mV1A, mV2A, mV3A and mV4A). Further along with the VHH-IgA construct, co-expression of Joining chain (J chain) and porcine secretory component (SC) in seeds, tetravalent dimeric (dV1A, dV2A, dV3A and dV4A) antibodies with VHH-IgA + J chain; and secretory IgA (sV1A, sV2A, sV3A

and sV4A) antibodies with VHH-IgA + J chain + SC were made.

All the seed-produced fusion antibodies prevented bacterial binding to gut villous enterocytes *in vitro* (Chapter 4 and 5). While *in vitro* results are an important primary step towards therapeutic development, in the long course of drug development, they need to be evaluated for *in vivo* functionality. Hence we evaluated in an *in vivo* feed-challenge experiment the potential of Arabidopsis seed-produced fusion antibodies in preventing F4$^+$ETEC bacteria from colonizing the small intestine.

Results and Discussion

Optimisation of the feed-challenge experiment

The experimental disease model as described by Cox *et al.* for evaluation of anti-F4$^+$ETEC immunotherapeutic upon challenge requires fasting period and deprival of water before inoculation of the bacteria via intragastric intubation (Cox *et al.*, 1991). This model ensures experimental infection of each animal, and has been used in several mucosal vaccination experiments (Van den Broeck *et al.*, 1999a; Van den Broeck *et al.*, 1999b; Verdonck *et al.*, 2004b) including evaluation of *in planta* produced FaeG-based vaccine to prevent F4$^+$ETEC infection (Joensuu *et al.*, 2006b). However, fasting is not suitable in evaluation of in-feed administration of seed-produced antibodies for prophylactic oral passive immunisation; as this would lead to a discontinuation in supply of oral therapeutic antibodies. It has also been observed that *ad libitum* feeding during challenge in this particular disease model generally leads to a lower shedding profile (Coddens and Cox, personal communication), we hence tried to optimise this challenge model. In addition to the feeding and fasting regimen it is also suggested that the feed composition, particularly the lactose content also influences the experimental F4$^+$ETEC infection (Melin and Wallgren, 2002). The F4$^+$ETEC have been shown to recognise specific glycans, and F4ac fimbriae specifically bind to the glycosphingolipids such as lactosylceramide and galactosylceramide expressed on erythrocytes and intestinal cells (Coddens *et al.*, 2011). Therefore, lactose and other structurally similar glycans in feed or its derivatives on gastric digestion could influence the binding and colonisation of

pig intestine after experimental F4⁺ETEC infection.

Lactose in varying concentration can be found in almost all commercially available starter-feed, and is highly preferred since it increases the palatability of starter-feed during transition from milk to solid feed and benefits weight gain (O'Doherty *et al.*, 2004; Pierce *et al.*, 2004). Since pure lactose is expensive, commercially whey-premixes are available as cheap source of lactose. Such whey-premix contains up to 6% whey powder and other essential vitamins. Our *in vitro* analysis of such a commercial whey-premix showed that increasing concentration of this premix higher than 2.5% has an inhibitory effect on F4ac fimbriae binding in ELISA with immobilised mucus receptors, while 1% whey-premix solution completely inhibits any bacterial binding to its villous enterocyte receptor in an *in vitro* villous adhesion test (Figure 6.1, a and b). Comparatively, the basic feed, devoid of any whey-premix does not present similar inhibitory effect and only marginally influences the anti-F4 antibody interaction with F4 fimbriae at 10% feed solution, which is the highest concentration tested (Figure 6.1, a).

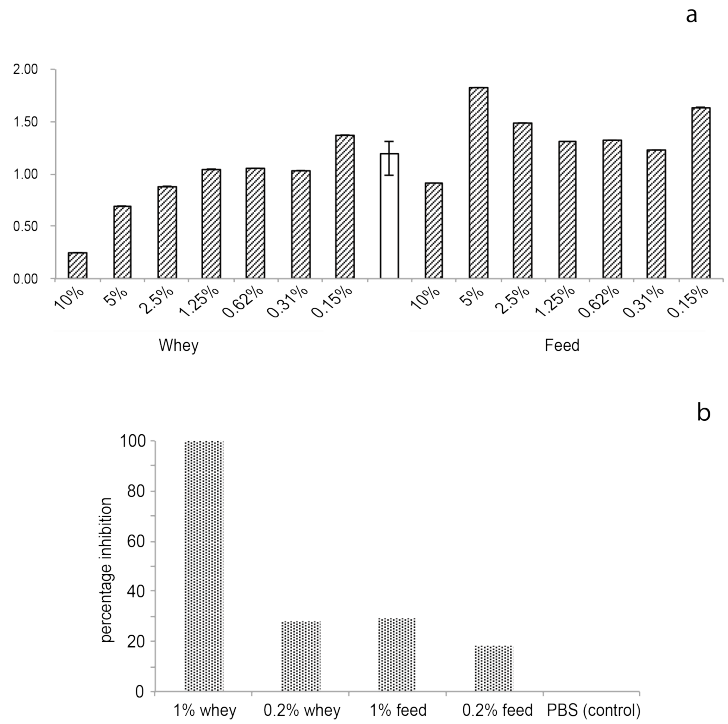


Figure 6.1: *In vitro* inhibition of whey containing premix in comparison to basic feed. As compared to soluble extracts made from basic feed without lactose source, soluble extracts made from whey (as lactose source) containing premix inhibits the F4 fimbriae interaction with anti-F4 antibody in a competitive ELISA (a). The extracts from whey containing premix even prevent F4⁺ETEC attachment to villous enterocytes (b).

In the villous binding test as well, the basic feed without lactose was about 75% less effective in preventing bacterial attachment (Figure 6.1, b). Though field experiences differ; as addition of lactose or whey does not inhibit bacterial infection and onset of diarrhoea (Fairbrother *et al.*, 2005), our *in vitro* evidence of molecular interference of whey-premix cannot be discounted while establishing a feed-challenge experimental model. Thus with an aim to have controlled and standardised infection (relatively similar, reduced variation) without deprival of food and water prior to challenge, the following modifications were tested.

The *in vivo* effect of two different basic feed formulations–

Feed with and without lactose were evaluated in combination with two challenge regimens

Ten piglets seronegative for antibodies against $F4^{+}$ETEC and F4ac receptor positive, as determined by the mucin4 gene polymorphism (Rasschaert *et al.*, 2007) assay were selected, and housed in two isolation units with 5 piglets in each group. Group 1 (piglets number 1-5) received the lactose-free customised feed, while group 2 (piglet no. 6-10) received commercial piglet starter feed, both these feeds were given *ad libitum*. All the piglets were challenged as previously described (Cox *et al.*, 1991) with the exception that they were not deprived of food and water. All the piglets were challenged on day 0, in addition 2 piglets per group (piglet no. 4&5, 9&10) were challenged also on day 1 with the same dose of bacteria (10^{10} bacteria). The faecal shedding of the challenge strain Gis26 (Cox and Houvenaghel, 1993) was noted every day from day 1 until day 8, confirmed by colony immunoblotting with anti-F4 monoclonal antibodies, and expressed as the log of bacteria present per gram of faeces in Figure 6.2.

A strong difference was not observed between the two feeds or the number of challenge, however to achieve uniform infection that might lead to a more typical inverted bell shape shedding profile (Figure 6.2), the 2 times challenge regimen in combination with the commercial feed (Figure 6.2) was chosen. Hence the disease model for feed-challenge experiment was optimised to– no fasting, challenging with the pathogenic strain on two consecutive days and the

commercial feed was used as basic feed in which antibody producing Arabidopsis seeds were added to formulate the prophylactic treatment feed.

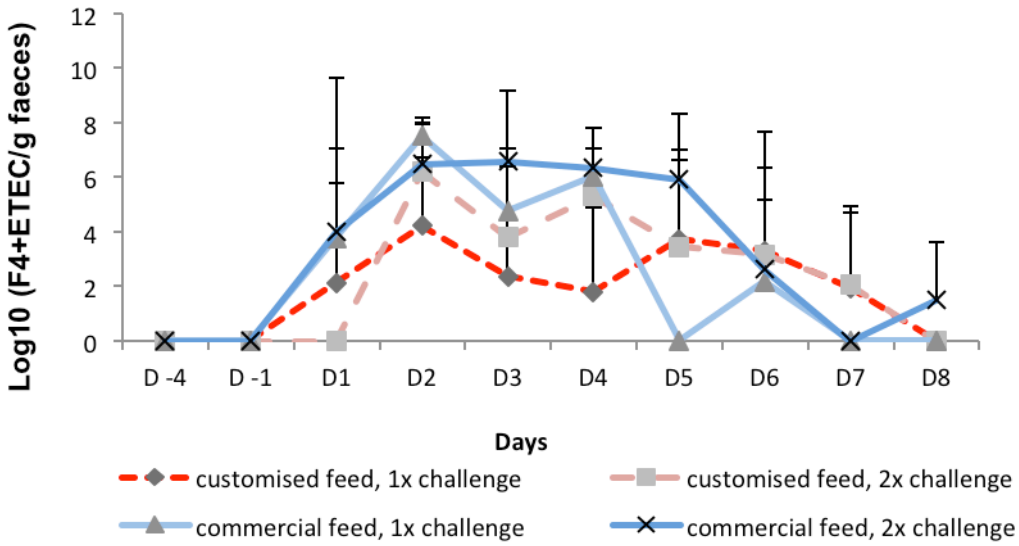


Figure 6.2: Optimisation of feed and challenge regimen by comparison of the F4+ETEC shedding profile. The error bars represent standard deviation within the experimental population.

Palatability of Arabidopsis seeds in piglet diet

Feed ingredients influence the taste, smell, digestibility and thus the general acceptance and palatability of a particular feed formulation. Addition of flavouring agents is suggested to influence the feed intake and a preferred pleasant flavour also eases the transition from milk to solid feed[16]. Along with the flavour, the basic feed composition, primarily the protein (followed by minerals) concentration, influences the feed to weight gain conversion ratio (FCR) (feed conversion ratio = mass of feed consumed / body mass gain in the given period of time) which is an important parameter for judging the feed influence on economic profit margin (Heo *et al.*, 2012; Kim *et al.*, 2012; Sola-Oriol *et al.*, 2011). Moreover in case of in-feed administration of a therapeutic protein, an increase or decrease in the palatability and acceptance of the feed can influence the final dose of the therapeutic protein administered.

[16] Vande Ginste J. "Palatability improves young piglets' feed intake" (http://alturl.com/rx6g6)

To the best of our knowledge the palatability of Arabidopsis seeds in piglet feed has never been assessed, possibly since apart from being a model plant for studying plant biology Arabidopsis is regarded as a weed. Arabidopsis seeds have about 40% crude fat, 20% crude protein; and to human olfactory senses the seeds have no strong odour but milled Arabidopsis seed flour bears a peanut butter-like scent. We noticed that addition of 10-20% of Arabidopsis seed flour (premixes) to piglet basic feed also changes the colouration of the piglet feed to a darker shade of brown however these effects diluted out at higher dilution (~2% inclusion rate).

Table 6.1: Arabidopsis seed (wild type seeds) flour at 2% inclusion rate in piglet feed was observed to be palatable

	Daily average feed intake (in grams)						Total weight gain in 6 days (in kg)	Feed to weight conversion ratio (FCR)
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6		
Starter feed without wild type Arabidospsis seeds	380 ± 40	405 ± 40	475 ± 70	530 ± 80	640 ± 100	0.700 ± 40	2.850 ± 0.29	1.10 ± 0.04
Starter feed with wild type Arabidopsis seeds	445 ± 40	510 ± 70	545 ± 100	645 ± 140	690 ± 140	0.725 ± 150	2.925 ± 0.57	1.23 ± 0.16

We assessed the palatability and FCR on adding Arabidopsis seed flour to piglet feed at 2% inclusion rate in a piglet feeding experiment. Eight piglets were weaned at 3 weeks of age and housed in individual feeding cages. Individual feeding cages enable exact determination of feed intake per day for each pig, however it has been generally noticed that piglets on an average consume more feed in this setup as compared to group housing. For the first week after weaning all piglets received creep feed, and on an average consumed 307 ± 51 grams of feed (FCR was 3.0 ± 1.8). Once the piglets were acclimatised to solid feed, from the second week onwards the feed for 4 of these piglets was changed to starter feed containing 2% Arabidopsis seeds while the remaining 4 control group piglets received regular starter feed. The daily feed consumption in week 2 increased each day for all the piglets, it was seen that the piglets readily accepted the 2% Arabidopsis seeds in the feed and this feed was comparatively palatable (Table 6.1). The control group on an average consumed about 522 ± 47 grams of feed per day, while piglets receiving 2% Arabidopsis containing feed on an average consumed 593 ± 99 grams of feed per day. The preference for the feed containing 2% Arabidopsis seeds could be due to the taste attributed by higher overall fat content in this feed. The results obtained showed that the piglets

accepted the Arabidopsis containing feed. The FCR was within 1.3, which is an average standard FCR reported for different feeds for piglets at this age17. These two factors encouraged the use and further evaluation of transgenic antibody producing Arabidopsis seeds in piglet feed, without the requirement of special feed flavouring or nutritive ingredients to maintain its palatability.

Feed-challenge experiment: evaluation of protection conferred by in seed made anti-F4$^+$ETEC fusion antibodies on experimental challenge

From the previous results we demonstrated that all formats of in seed produced anti-F4$^+$ETEC antibodies i.e. VHH-IgG in its monomeric and VHH-IgA in its monomeric, dimeric-tetravalent and in sSIgA formats are functional *in vitro* (Chapter 4 and 5). These antibodies were designed and produced in plants with an aim to protect upon oral passive immunisation against F4$^+$ETEC related PWD. We evaluate this fitness and functionality of the seed produced antibodies on oral administration in the *in vivo* optimised feed-challenge experiment. We evaluated both the antibody formats VHH-IgG and VHH-IgA in their own merit by using an oligomeric cocktail of each.

To select the treatment dose for therapeutic feed formulation, our calculations were drawn from the previous results of De Geus *et al*. (1998) where in a neonatal challenge model they demonstrated the protection conferred by monoclonal anti-F4$^+$ETEC antibodies (murine IgG1) at a concentration of 8 mg/100 ml of milk. Based on the consumption of milk by neonatal piglets until weaning (4 weeks of age) and the weight gain to feed consumption ratio during the first and second week after weaning, we estimated that if not weaned, 4-5 weeks old piglets would consume a minimum of one litre milk per day. Thereby to attain equivalent protection **~80 mg of antibody would be needed per piglet per day.**

The VHH-IgG oligomeric cocktail contained approximately equal parts by weight of the 4 VHH-IgG antibody producing Arabidopsis seed stocks, i.e. V1G seed stock expressing antibody up to 2% of seed weight, V2G seeds stock expressing antibody up to 3% of seed weight, V3G and V4G seeds each expressing antibody up to 0.4% of seed weight (Chapter 4). Thus the antibody content in the pool of oligomeric VHH-IgG cocktail was about 1.4% of seed weight (average of the 4

accumulation, also experimentally verified in a sample protein extract made from the oligomeric pool of VHH-IgG seeds). Similarly the VHH-IgA oligomeric cocktail contained approximately equal parts by weight of the 12 antibodies producing Arabidopsis seed stock, i.e. the 4 monomeric IgAs (mV1A, mV2A, mV3A and mV4A), the 4 tetravalent-dimeric IgAs (dV1A, dV2A, dV3A and dV4A) and the 4 sSIgAs (sV1A, sV2A, sV3A and sV4A). Since the concentration of each of these 12 IgA producing line was similar about 0.2% of seed weight (Chapter 5), the concentration of functional antibody (all 3 formats) in the pooled IgA seeds remains about 0.2% of the seed weight. Thus every **gram of VHH-IgG oligomeric cocktail of Arabidopsis seeds contained 14 mg of VHH-IgG antibody; while a gram of IgA oligomeric cocktail of Arabidopsis contained 2 mg of antibody**. Since a substantial portion of the VHH-IgA treatment cocktail contains the tetravalent constructs (dIgA and sSIgA), we opted to use 4 times less VHH-IgA dose (i.e. 20 mg) as compared to the VHH-IgG dose (80 mg) in the feed-challenge experiment.

Hence in our feed-challenge experiment we chose three treatment groups receiving (i) **80 mg of VHH-IgG** per day, (ii) **20 mg of VHH-IgA** antibody per day, and to evaluate the effect of equivalent amount of VHH-IgG, we included the third treatment group receiving **20 mg of VHH-IgG** per day. The fourth group was the negative control (NC) group, which received wild type Arabidopsis seeds equivalent in proportion to Arabidopsis seeds in VHH-IgG-80 group (Table 6.2). To attain these desired daily doses, the respective treatment feed containing milled Arabidopsis seeds was formulated in accordance to the antibody expression level in the oligomeric cocktail and the average feed consumption per pig per day.

The daily feed intake of piglets from the age of 4 to 6 weeks when housed in group in a pen varies from 300-400 grams[17], and in general is low at 4 weeks of age and gradually increases (feed intake data provided from the previous experiments courtesy of Dr. Coddens performed at Veterinary Faculty, Ghent University and by Dr. Millet from feed intake experiments performed at the Institute for Agricultural and Fisheries Research, Animal Sciences Unit, Ghent).

[17] http://www.thepigsite.com/stockstds/18/daily-feed-intake

From the expression levels of respective antibodies in the two seed oligomeric pools and taking into account that **the piglets consume 300 grams of feed per day** the experimental feed dose was formulated.

Table 6.2: Experimental feed formulation of the 4 respective groups.

Groups	Dose	Number of piglets	Total feed prepared in kg	Proportion of Arabidopsis seeds in feed	Proportion of flax seeds in feed
VHH-IgG-80	80 mg/300 gram feed)	7 pigs	21.36	1.9% (0.40 Kg)	1.4% (0.3 Kg)
Negative control		7 pigs	21.36	1.9% (0.40 Kg)	1.4% (0.29 kg)
VHH-IgA-20	(20mg/300 gram feed)	4 pigs	15.3	3.3% (0.50 Kg)	n.a.
VHH-IgG-20	(20mg/300 gram feed)	3 pigs	10.5	0.48% (0.05 kg)	2.82% (0.3 kg)

The average daily feed consumption of piglet at 3 – 4 weeks of age is about 300 grams. (n.a. stands for not applicable)

The concentration of VHH-IgG antibody was 14 mg in every grams of crushed VHH-IgG Arabidopsis seed flour. Therefore, to formulate a dose 80 mg antibody per pig per day in VHH-IgG-80 feed, 5.71 grams of Arabidopsis flour was mixed in every 300 grams of basic piglet feed i.e. at inclusion rate of 1.9%. Further, to dose 20 mg of VHH-IgG per pig, same VHH-IgG Arabidopsis seed flour was added to basic feed at 4 times less inclusion rate of 0.48%. Similarly since the VHH-IgA antibody concentration was 2 mg/gram of crushed Arabidopsis VHH-IgA flour, to dose 20 mg per pig per day, 10 grams of VHH-IgA Arabidopsis flour was mixed in every 300 grams of basic feed, i.e. at an inclusion rate of 3.3% (Table 6.2). The negative control group piglets received feed with 1.9% wild type Arabidopsis seed flour (equivalent to VHH-IgG-80 feed). Each of these 4 experimental feed formulations thus had varying concentration of Arabidopsis seeds; this could have had an effect on the nutrition of each feed. So as to maintain nutritive homogeneity, flax seed meal was added. The flax seed composition is relatively similar to Arabidopsis about 41% fat, 20% crude protein, 28% crude fiber, 7.7% moisture and 3.4% crude ash (Morris, 2007)[18]. Further the milled flax seeds do not have any strong odour and can be commercially procured. The feed VHH-IgA-

[18] From flaxcouncil.ca

20 contained the highest amount of Arabidopsis seeds i.e. 3.3% inclusion rate; hence to equalize the nutritive content flax seed meal was added to the other three experimental treatment feeds (Table 6.2). Additionally to enable similar nutritional balance throughout the experiment, the piglets were fed with feed containing 3.3% crushed flax seeds (flax-feed) both before and after the challenge regimen (see scheme, Figure 6.3).

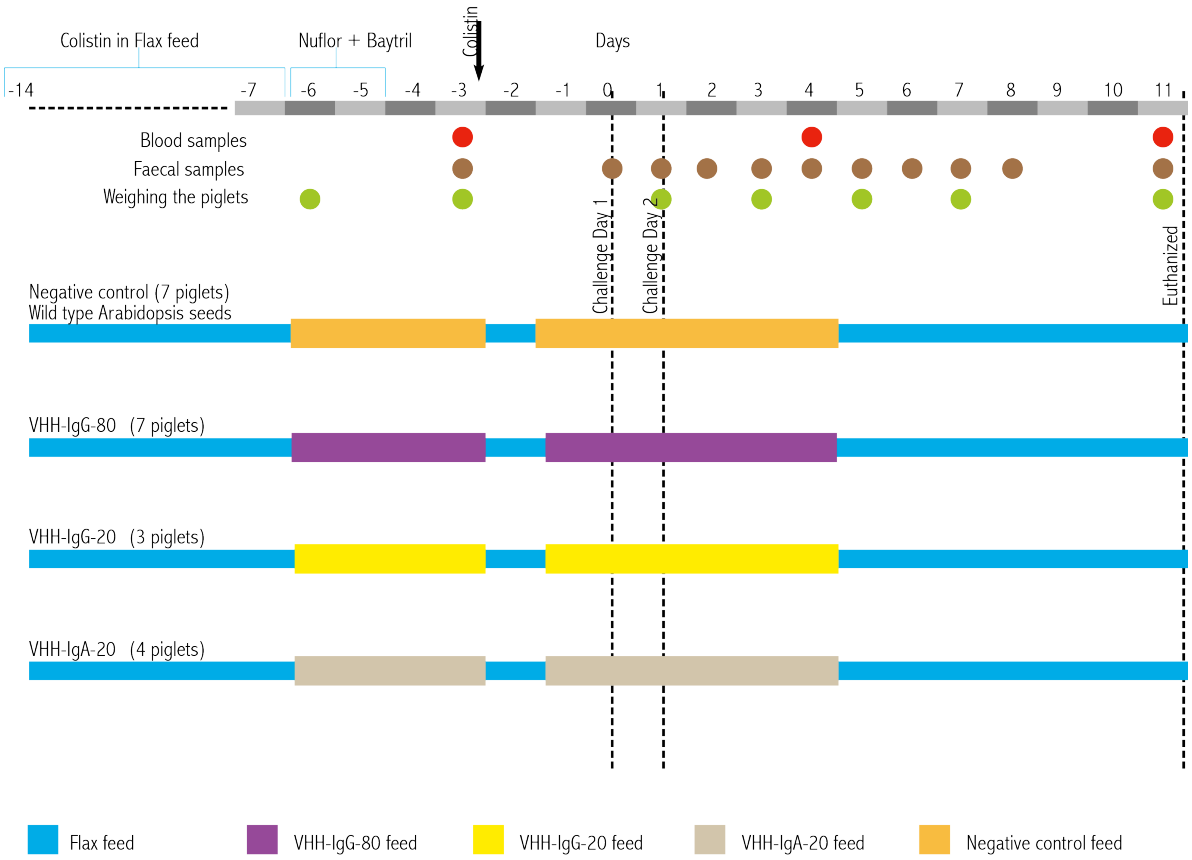


Figure 6.3: Schematic representation of the *in vivo* experiment. All the events in the feed-challenge experiments have been depicted in the figure with reference to the time line (top), time point of sample collection (blood and faeces) and weighing of piglets is denoted with circles. The feeding regimen for the four groups is indicated with bars, with feed key at the bottom.

To perform the optimised feed-challenge infection, twenty-one anti-F4$^+$ETEC seronegative piglets, all genotypically heterozygous for the mucin4 gene polymorphism were selected (F4 receptor positive). Prior to challenge, these piglets were housed in 4 individual units, 7 of which were housed in the VHH-IgG-80 group, 4 in VHH-IgA-20 group, 3 in VHH-IgG-20 group and the remaining 7 piglets constituted the negative control group. The feeding regimen, days of challenge (2 times), days of weighing, blood and stool collection time points are

schematically represented in Figure 6.3, where the first day of challenge is regarded as day 0. One day before challenge the flax feed in each pen was replaced with their respective experimental feed; 300 g of respective feeds per pig (plus about 50 grams to account for spillage) was provided in the common feeding vats per pen, until day 4.

The daily rectal-faeces samples collected per piglet were plated on blood agar plates with 1 mg/ml streptomycin and 20 µg/ml tetracycline to evaluate the daily shedding of the F4+ETEC challenge strain– Gis26R^{strep}[19]. These colonies of Gis26R^{strep} isolated from faeces upon challenge, produced marked haemolysis, the specific count of which could be dually confirmed with an immunoblot assay with anti-F4 monoclonal antibodies. An overview of the individual shedding patterns is given in Supplementary Table 6.ST1. All the piglets shed high titers of the challenged F4+ETEC strain immediately after first day of challenge. The negative control group on an average shed bacteria higher than 6 $\log_{10}$ per gram of faeces until day 4, after which the bacterial shedding gradually declined until day 8 (Figure 6.4).

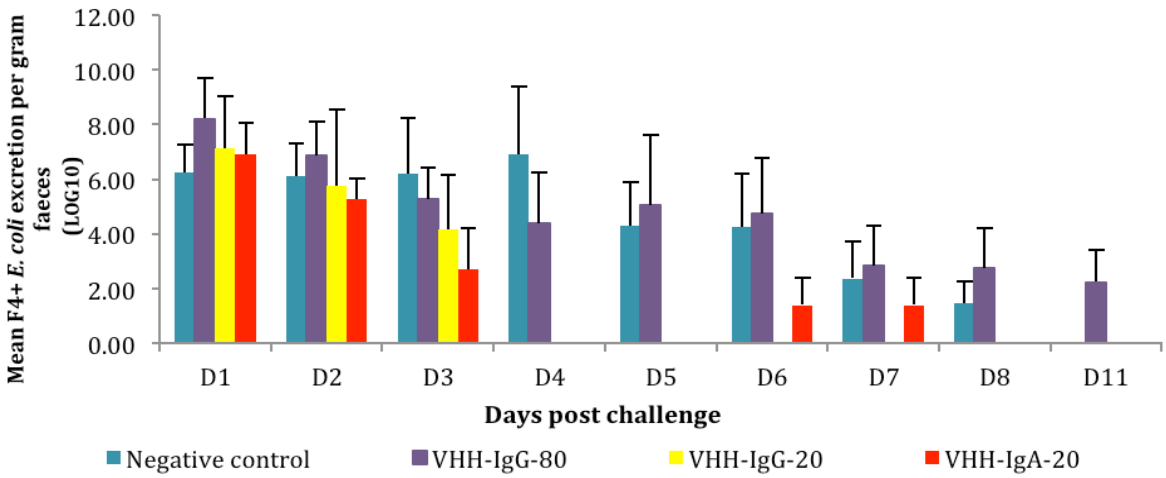


Figure 6.4: In feed provided VHH-IgA rapidly decreases bacterial shedding post experimental infection as compare to other groups. In the group fed with feed supplemented with seeds containing VHH-IgA (VHH-IgA-20, n=4), the bacterial shedding rapidly decreased post experimental infection as compared to other groups fed with feed supplemented with wild type seeds (Negative control, n= 7) or supplemented with seeds containing VHH-IgG (group VHH-IgG-20,n=3 and VHH-IgG-80, n= 7). The error bars represent standard deviation within the group. The evolution of bacterial shedding in the course of time within the group VHH-IgG-80, VHH-IgA-20 and the Negative control group was significant (p = 0.019). The experimental feed was provided till day 4.

[19] N.B. Gis26 is a field isolate, serotype O149:K91: F4ac, producing the heat-labile enterotoxin (LT+) and heat-stable enterotoxin types a and b (STa+, STb+) which was progressively scaled up on higher concentration of streptomycin, to produce strain resistant to 1 mg/ml streptomycin called Gis26R^{strep}

Maintenance of the high shedding in the initial 4 days is suggestive of the bacterial multiplication in the gut and possible colonisation in the small intestine, which gradually over time declined after day 4 until day 8. Similar trend of prolonged, and gradually decline in shedding profile is usually seen in negative controls in oral immunisation and challenge experiments with $F4^+$ETEC in a piglet model (Snoeck *et al.*, 2003). In contrast to the negative control group, the in-feed administered **VHH-IgA** seemed to prevent the bacteria infection, as immediately after the challenge the bacterial shedding was rapidly decreased by day 4 to below detection level of less than 100 bacteria per gram of faeces (Figure 6.4). The VHH-IgA-20 feed like all the experimental treatment feeds was provided until the day 4, there after the feed was changed to flax-feed. Interestingly, we noticed a single piglet (no. 21) showed marginal shedding of ~100 bacteria per gram of faeces (2 $\log_{10}$/ gram of faeces) on day 6 and 7 (Supplementary Table 6.ST1). After this period, $F4^+$ETEC could no longer be detected in faeces examined on day 8 or on day 11, for any of the piglets in this VHH-IgA-20 group.

On day 11, all the piglets were euthanized and the intestinal and caecum content for all piglets was also plated on selective medium. Here, we could also detect 4.2 $\log_{10}$ (1.6×10^4) bacteria per gram caecum content and 3.5 $\log_{10}$ (3.2×10^3) bacteria per gram of ileum content only from piglet no. 18 (VHH-IgA group) and non of the other 20 piglets.

The ileum, caecum, colon and rectum are physiologically very different. The physical nature of its contents are dissimilar, particularly the concentration of solid fibrous matter and water content making it difficult to compare the bacterial load between these different compartments of the intestine. Nevertheless, the detection of this miniscule secondary peak 3-4 days after stopping the VHH-IgA-20 treatment feed and further isolation of bacteria in the gut on euthanizing suggests that a longer treatment, minimum up to 10 days post challenge might be ideal to reduce the overall infection pressure.

Similar rapid clearance of the bacteria by day 4 after challenge was also seen in the **VHH-IgG-20** group, however not in case of the third treatment group **VHH-IgG-80**. The shedding profile observed for these 7 piglets of VHH-IgG-80 groups

showed maintenance of high infection pressure, and clearly indicates that the VHH-IgG at 80 mg dose per day failed to protect the piglets (Figure 6.4). The prolonged higher rate of shedding as compared to negative control suggests that the VHH-IgG treatment at the dose of 80 mg per day was rather detrimental and synergistic for F4$^+$ETEC infection than protective.

These results at the first instance suggested that equivalent dose of 20 mg of VHH-IgG antibody per day per pig, to be equally effective in bacterial clearance as 20 mg of VHH-IgA antibody cocktail per day per pig (Figure 6.4). However the quantitative determination of the F4R status performed post-mortem by villi binding test, revealed that out of three piglets in this group, two had very few F4 receptors (Supplementary Table 6.ST1). In this test the average number of F4$^+$ETEC bound per 250 µm of the villous enterocytes surface gives a relative account of F4R receptors expressed (Coddens *et al.*, 2009). Less than 5 bacteria per 250 µm of villous surface is regarded as F4 receptor negative piglet, 5-30 bacteria/250 µm villous surface is regarded as moderate distribution of F4 receptor while more than 30 is regarded as high expression of F4 receptors. Within the group VHH-IgG-20, an average of 7.5 and 5.75 bacteria per 250 µm of villous length was observed in piglet no. 15 and 17 respectively, leaving just piglet no. 16 with an average of 36.25 bacteria per 250 µm of villous surface as the only susceptible piglet in this group. Piglets with no or relative absence of F4 receptors are known to be naturally insensitive to F4$^+$ETEC infection, such piglets shed very low bacteria and are protected even on experimental challenge (Geenen *et al.*, 2004; Geenen *et al.*, 2007). This situation within the VHH-IgG-20 group would lead to an overall diminished infection pressure in the pen since 2 of 3 piglets were relatively insensitive to F4$^+$ETEC infection. With this knowledge it's difficult to attribute the protective effect of VHH-IgG-20 feed bearing antibody for the reduction of ETEC bacterial shedding in piglet 16 by day 3. Hence, we decided to exclude this group from any of our comparative statistical group analysis. But at the same time one cannot discount the putative role of VHH-IgG-20 feed against F4$^+$ETEC infection in susceptible piglets. Hence we report the finding of this group as preliminary results.

Piglets in the other group on an average had moderate expression of F4R. In the

4 piglets of the VHH-IgA-20 group (Supplementary Table 6.ST1), approximately 32 ± 8 bacteria adhering per 250 µm of the villous surface was observed; thus ruling out any alleged possibility of F4 receptor negative status for the rapid clearance of challenge bacteria in VHH-IgA-20 group. In case of the third treatment group VHH-IgG-80, the 7 piglets an average had 27 ± 6 bacteria attached per 250 µm of the villous surface. Also the negative control group on an average had moderate expression of F4R. Thus in theory, except VHH-IgG-20 group, the moderate receptor positivity in the other three groups would enable achieving high infection pressure in the respective group, hence the corresponding bacterial shedding results were accountable for these 3 groups.

IgG as candidate for passive immunisation has been suggested since decades, humanised IgG antibodies for intravenous passive immunisation are even clinically administered (Chapter 2). Given the success of these intravenous IgG based passive immunisation therapy, it is often assumed that IgG that survive the gut would render similar protection. However our negative results from the VHH-IgG-80 group suggest contrary to traditional believes; and hints that IgG might not always be the best for oral passive immunisation, especially in the light of recent research on the Fc neonatal receptor (FcRn). Stirling *et al*. (2005) while studying porcine neonatal Fc receptor noticed almost a promiscuous transport of orally administered bovine IgG in to piglet blood circulation, through the medium of porcine neonatal Fc receptor (FcRn) (Stirling *et al.*, 2005). FcRn are expressed in the gut of juvenile as well as adult pigs and thus in affirmation with Baker *et al*. (2009) the receptor is indeed “not so neonatal” as the name suggests (Baker *et al.*, 2009). The faster seroconversion (Figure 6.5) and prolonged bacterial shedding observed in our study, could be due to the innate interaction of porcine IgG3 Fc of the VHH-IgG construct with the porcine FcRn expressed in the gut. Given the highest predicted affinity of binding between porcine FcRn and IgG3, rather than other IgG subclass, this interaction in gut would not be a distant likelihood (Butler *et al.*, 2009). IgG does not necessarily has to be bound to an antigen for its interaction with FcRn, thus the abundant IgGs in VHH-IgG-80 in the gut would have extensively occupied the FcRn. This FcRn bound VHH-IgGs displaying their fimbriae binding paratopes might have facilitated bacterial attachment and colonisation.

Arguably, this synergistic effect of VHH-IgG-80 could be due to an overdose effect and a lower dose might be protective, or there might be another mechanism at play. Nevertheless, further insight into this hypothesis would be interesting in not just evaluating the reason of this observed effect on shedding of bacteria but more importantly it will also pave way for immunisation strategies to elicit gastric mucosal immune response together with systemic immunity, by exploiting the role of FcRn mediated transport across gut epithelium (Stirling *et al.*, 2005).

Seroprofile corroborates shedding profile

The overall shedding profile of each of the treatment groups correlated to the observed rise of anti-F4 fimbriae antibodies observed in the blood samples taken on day -3, day 4 and day 11 for each respective group; providing dual confirmation of the efficacy of each treatment and casting more light on its mechanism (Figure 6.5). The rise in serum IgM levels as early as the 4th day post challenge is indicative of this challenge being the primary infection and immune response to F4^{+}ETEC bacteria (Figure 6.6). In various previous challenge experiments rise in anti-F4 fimbriae IgG in serum has been detected in the control group on an average by the 7th day post challenge (Snoeck *et al.*, 2003). In agreement with the previous studies, high titers of serum anti-F4 fimbriae IgG were detected in serum collected from the negative control group, and also for both the VHH-IgG treatment groups later in the experiment on day 11 (Figure 6.6). In comparison with these three groups, the evolution of immunoglobulin seroconversion rate observed for the 4 piglets in the VHH-IgA-20 group over time was much lower at each time point (at day -3 *p=0.018,* at day 4 *p=0.059,* at day 11 *p=0.033*) (Figure 6.5). This suggests that the in seed made VHH-IgA antibodies at its 20 mg dose per day provided passive protection at the gut mucosal surface by preventing the interaction of the pathogenic bacteria with host cells and avoiding priming of the immune system.

Contrary to the VHH-IgA the feed formulations VHH-IgG-80 failed to achieve such passive protection, as evidently the respective seroconversion rate over time was comparatively high. The single susceptible piglet from the VHH-IgG-20 group also seroconverted at a faster rate, however the average (as plotted in the

graph) was approximately similar to the negative control. Whereas, for the VHH-IgG-80 group a higher average titer on day 11 and early rise in antibody titer, was noticed on the day 4. The overall higher titer of VHH-IgG-80 group could be due to the higher bacterial infection, which corroborates by higher bacterial shedding in the group.

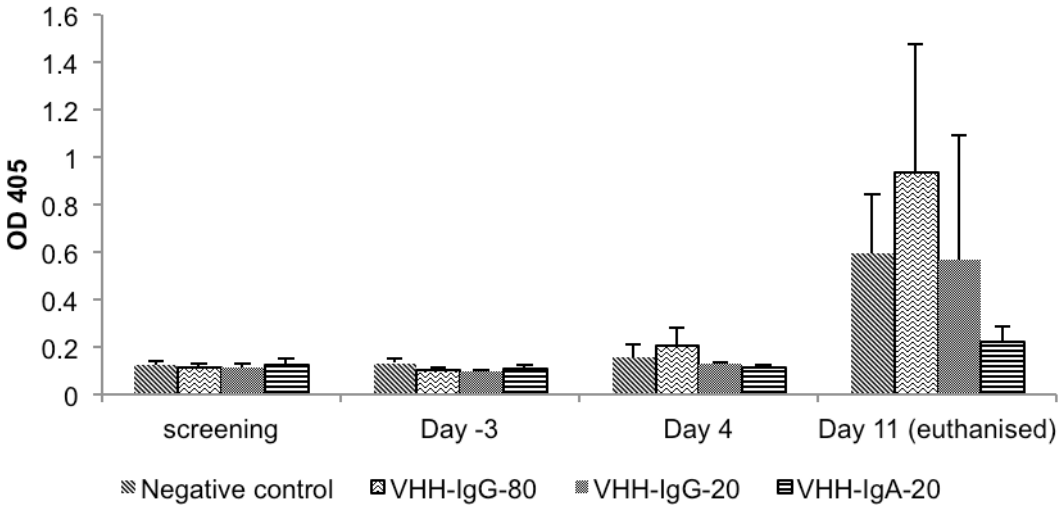


Figure 6.5: Rate of seroconversion (all antibody classes) post challenge in the 4 experimental groups. The anti-F4 fimbriae antibodies (all classes) in the serum of the piglets were detected by ELISA with polyclonal anti-Pig immunoglobulin antibodies. The error bars represent standard deviation within the respective groups. The evolution of seroconversion between the group VHH-IgG-80, VHH-IgA-20 and the negative control from day -3 of challenge till they were euthanised i.e. day 11 was significant (*p = 0.003).*

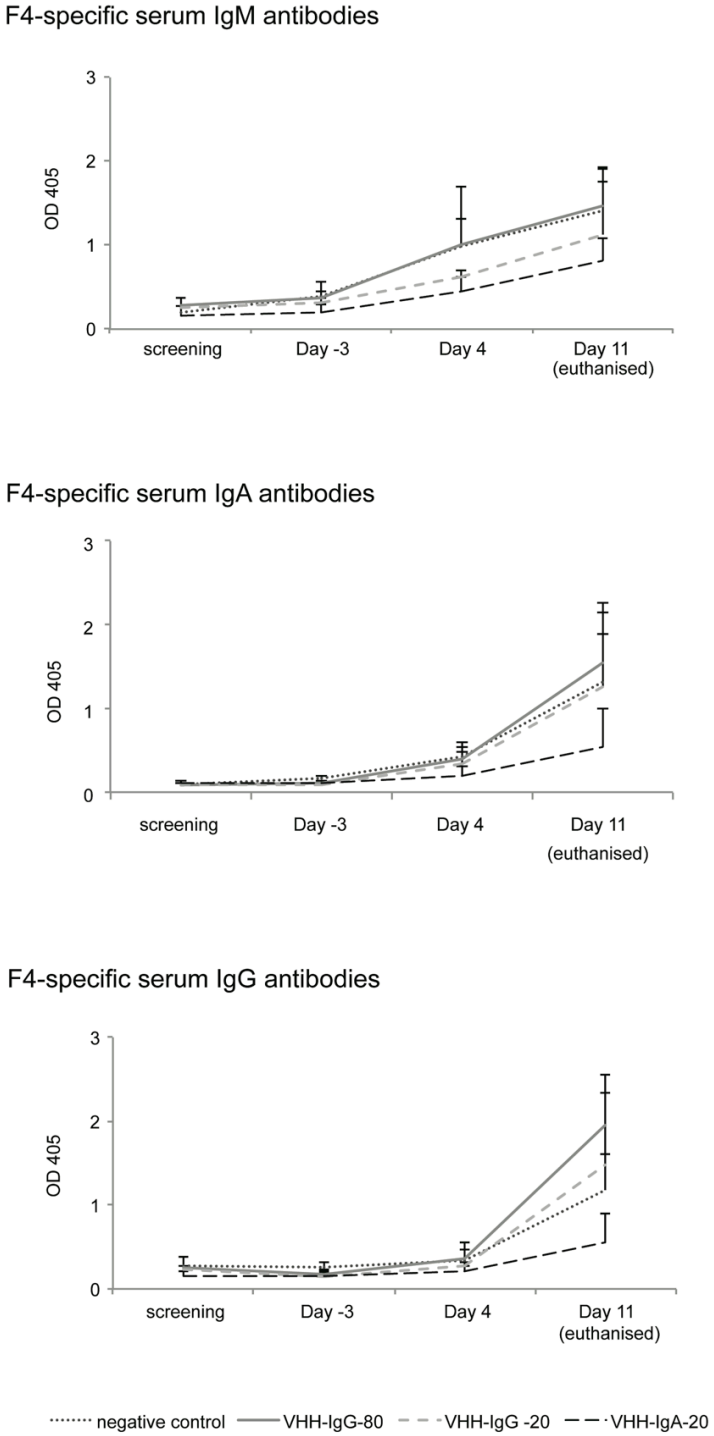


Figure 6.6: Comparison of serum immunoglobulin isotype response post F4⁺ETEC challenge in the 4 experimental groups over time. The piglets were challenged on day 0 and day 1. The earliest rise of serum anti-F4 fimbriae IgM level by day 4 in all piglets is indicative of F4⁺ETEC challenge being the primary immune response, further the serum anti-F4 fimbriae IgG and anti-F4 fimbriae IgA levels rose after day 4 as detected on day 11. The evolution of anti-F4-IgM between the group VHH-IgG-80, VHH-IgA-20 and the negative control before challenge (day -3) till the day of euthanasia i.e. day 11 showed tendency of being significant (top), while the difference was significant at day -3 (p = 0.044) and day 11 *(*p = 0.025). The evolution of anti-F4-IgG (bottom) and anti-F4-IgA (middle) between the group VHH-IgG-80, VHH-IgA-20 and the negative control from day -3 till the day they were euthanised i.e. day 11 was significant (*$p = 0.00$ and $p = 0.01$* respectively).

Effect of the in feed prophylaxis on the weight gain of piglets

The pattern of bacterial shedding and the seroconversion rate are important parameters for assessing the protection conferred by the in-feed produced anti-F4⁺ETEC antibody treatment. However, for the porcine industry additionally to the efficacy of treatment, the piglet weight gain parameter is of utmost importance since it bears a direct relation to economic benefits. Piglets suffering from post-weaning diarrhoea usually have a worse feed to weight conversion ratio, in comparison to healthy piglets.

In our study, we found that the average weight gain for the negative control piglets (from day -3 to day 11) was low. On the contrary, the weight gain in all three experimental feed treatment groups was higher than the negative control (Supplementary Table 6.ST2). The average weight gain was highest for the VHH-IgG-20 group and like the rapid bacterial clearance seen in this group; the weight gain could also be the effect of F4R related insensitivity to F4⁺ETEC infection. Hence, except this group the daily weight gain of the remaining piglets in the three groups was calculated from the weight measured during the experiment (see scheme, Figure 6.3).

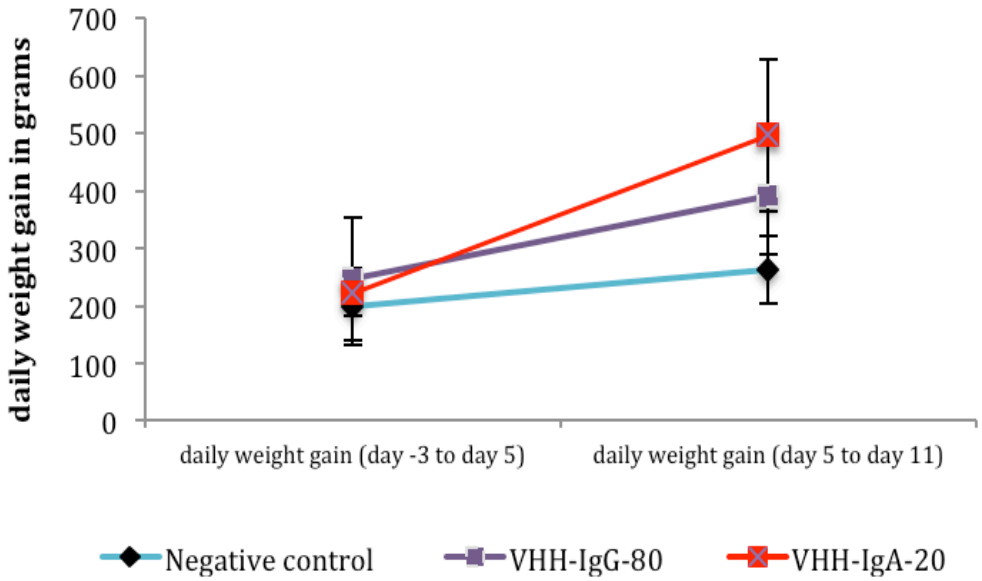


Figure 6.7: Comparative weight gain during (day -3 to day 5) and after the experimental diet (day 5 to day11) in the three groups. The bars represent standard deviation in weight gain from the average daily weight gain calculated, in grams. This observed evolution of weight gain for the three groups over time was highly significant ($p = 0.006$).

The daily weight gain of all the piglets in the three groups (negative control, VHH-IgG-80 and VHH-IgA-20) around the challenge period while on Arabidopsis seed flour containing diet was reasonably similar (day -3 to day 5). However after changing this feed to the basal diet (Flax feed) from day 5 to day 11, piglets previously on VHH-IgA-20 feed and VHH-IgG-80 feed had a higher daily weight gain, in comparison with the negative control (Figure 6.7). Understandably, the high weight gain for the VHH-IgA-20 piglets can be associated to protection against $F4^+$ETEC challenge conferred by the in-feed VHH-IgA antibodies. However, higher weight gain in VHH-IgG-80 group, in comparison with the negative group cannot be explained by the same reasoning. The weight gain in this group in spite of failure to achieve protection with VHH-IgG antibodies, nudges for further evaluation of this group and the FcRn hypothesis.

The VHH-IgG-80 feed though had a positive effect on weight gain; the average weight for the VHH-IgA-20 group still remains comparatively higher (Figure 6.7). In field conditions, both in large scale porcine farms as well as small scale subsistence farming, the ability of a therapeutic feed to rapidly limit the bacterial shedding helps in decreasing the overall infection pressure within the herd. The VHH-IgA prophylaxis in this study shows sign of such reduced overall infection pressure, making it ideal for a large-scale prophylaxis in porcine farms, to curtail outbreak of the highly contagious $F4^+$ETEC bacteria.

In this study we have demonstrated the potential of in seed produced customised antibodies in preventing $F4^+$ETEC infection and its positive effect on weight gain, which is promising for avoidance of economic losses to porcine industry. Further, production of these VHH-IgA antibodies in the seeds of crops like soybean, which can be bulk produced with the farming infrastructure, would enable cost effective anti-$F4^+$ETEC feed based prophylaxis.

Experimental procedures:

Bulk production of anti-F4+ETEC antibody expressing seeds and experimental feed formulation

Upscaling of the Arabidopsis seeds

Homozygous T3 seeds of the anti-F4+ETEC antibody producing lines were sown directly on jiffies, while for high expressing dIgA and sSIgA plants (dV1cA27, dV4A2, sV1cA36, sV1A8 and sV2A8) with multiple loci insertion, seeds were sown on kanamycin + PPT bearing selection plates and resistant plants were transferred to jiffies. The jiffies were placed in 51 pot-tray system (Araflat), in the green house with conditions as 16 hours of light/8 hours of darkness, 21°C and 55% relative humidity. Plants were watered weekly until the siliques started yellowing (6 weeks), 2-3 weeks later when the siliques dried completely the trays were harvested using a custom designed contraption nicknamed 'veiled-bin'. This is basically a huge metallic vessel onto which a bag made of nylon mesh is harnessed. Complete trays were inverted into the veiled-bin, the seeds were collected at the bottom of the vessel while the other dried biomass and jiffies were retained in the nylon mesh bag.

Feed formulation

The harvested Arabidopsis seeds were milled using a coffee grinder (Moulinex). Firstly the Arabidopsis seeds were flash frozen in liquid nitrogen, and every time ~ 50 grams of frozen seeds were added into the chilled metallic grinding bowl of the coffee grinder and milled for 1 minute. Precisely weighed Arabidopsis seed powder was then mixed with the calculated amounts of milled flax seed and piglet starter feed (Voeders van Huffel, Nevel, Belgium) as mentioned in Table 6.1 with Braun hand held kitchen mixer to make pre-mix containing 10x concentrated Arabidopsis seeds powder. This pre-mix was then diluted with starter feed and mixed using industrial feed mixer (ILVO) to obtained a homogenous mixture with the final desired concentration of crushed Arabidopsis seeds.

For the palatability test, the effect of adding 2% milled Arabidopsis wild type (Col 0) seeds in the feed of piglets was evaluated. And for the latter *in vivo*

challenge experiment, varying concentrations of transgenic Arabidopsis seeds determined by the dose of antibody were milled and fed to the piglets (Table 6.2). The maximum concentration of transgenic Arabidopsis seeds was 3.3% for the IgA category. In all the other formulations where the transgenic Arabidopsis seeds were less than 3.3 % of the feed, milled flax seeds were added to the feed to make up the equivalent 3.3% proportion and thus maintain nutritive homogeneity in all the feed formulations. Before and after the treatment regimen (Figure 6.3) all the piglets were provided Flax feed, which contained 3.3% flax seed mill and no Arabidopsis seeds.

Evaluating the palatability of Arabidopsis seeds in piglet diet

Before the *in vivo* feed trial, it was necessary to assess the palatability of Arabidopsis seeds in the feed of young piglets. Hence 8 piglets were weaned, and housed in individual feeding cages for 2 weeks. Each piglet received 1 kilogram of feed every morning which they could eat *ad libitum* over the whole day. Any leftover feed from the previous day was collected and weighed to determine the daily feed consumption. For the first week all the 8 piglets were given identical creep feed to acclimatise all the piglets to solid feed, once acclimatised in the second week the feed for 4 piglets was replaced with piglet starter feed (ILVO) as control group and other 4 pigs received the same starter feed supplemented with 2% milled Arabidopsis wild type Col 0 seeds. The consumption pattern of all the piglets was compared at the end of two weeks and the influence of adding 2% Arabidopsis seeds on the consumption of feed was evaluated.

***In vivo* evaluation of protection on feeding seed produced anti-F4$^+$ETEC antibodies and subsequent experimental F4$^+$ETEC infection**

Selection of piglets

Farms with relative absence of the F4$^+$ETEC infection were identified. The suckling piglets per sow were screened for anti-F4$^+$ETEC seronegative status by F4-specific ELISA and such seronegative pigs were further evaluated for their susceptibility by genotyping mucin4 receptor by RFLP (Rasschaert *et al.*, 2007), as follows.

Blood sampling and serum processing**:** About 5 ml blood was withdrawn from Jugular vein of the piglets from conventional Belgian porcine farm, at 3 days of age and then a part of this was aliquoted in microcentrifuge tubes pre-dispensed with 10% EDTA as an anti-coagulant, remaining blood was allowed to clump by maintaining at 37°C for 1 hour, and the serum fraction was taken. This serum fraction was further centrifuged at 20,800g for 10 minutes at 18°C to pellet any blood clumps, and obtain a clear serum fraction. This supernatant was heat inactivated for 56°C for 30 minutes and treated with kaoline. For the latter, one part serum was added to 4 part kaoline solution, vortexed well, and maintained at room temperature for 30 minutes, centrifuged for 10 minutes at 14,000 rpm at 18°C; the clear defatted serum fraction was stored at -20°C.

Screening seronegative piglets via F4-specific ELISA

The maxisorb ELISA multititer plates were coated for 2 hours at 37°C with 100 µl of anti-F4 monoclonal antibody (Laboratory of Immunology, Ghent University) dissolved in PBS at a concentration of 1 µg/ml. Post 2 hour, the plates were decanted, tapped on absorbent paper, and 300 µl of 0.2% Tween80 was added as blocking agent and the plates were incubated at 4°C overnight. Subsequently the plates were washed 4 times with 0.2% Tween20 in PBS, to this, 100 µl of purified F4 fimbriae (25 µg/ml) in dilution buffer (3% bovine serum albumin in PBS) was added and incubated for 1 hour at 37°C. After incubation the plates were washes as before and serum samples diluted 4x in dilution buffer were added in duplicate. At the same time, seronegative and seropositive reference sera were added as negative and positive control respectively. After another hour of incubation at 37°C, the plates were washed, 100 µl of anti-porcine goat polyclonal antibody conjugated to HRP diluted 1:5000 (Bethyl) in dilution buffer was added to each well and the plate was maintained at 37°C for one hour. Finally, after washing, 50 µl of substrate ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) was added and OD was measured at 405 nm every 5 minutes for 30 minutes. The OD of the piglet serum samples was compared to the controls and the seronegative piglets were selected.

Genotyping of sero-negative piglets expressing F4+ETEC receptor

Piglets seronegative for anti-F4+ETEC antibodies were screened for presence of F4ac receptor (F4R) via the non-invasive mucin4 polymorphism based PCR-RFLP assay as described by Rasschaert *et al.* based on the patent WO2004/048606-A2. The genomic DNA was extracted from the blood cells in the uncoagulated blood fraction with 10% EDTA as anti-coagulant. The nucleated blood cells were pelleted by centrifugation for 1 minute at 3800 g, the supernatant was discarded and the cells were washed 2 times with PBS each time maintaining the same centrifugation parameters. To the washed cells, 200 µl of proteinase K buffer (50 mM KCl, 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5% Tween20, pH 8.3) with 2 µl of proteinase K (Gibco) was added, and incubated at 37^0C for 1 hour, the enzyme was then deactivated by incubation at 95°C for 10 minutes. Five µl of this crude mixture containing the genomic DNA was used in a 20 µl volume PCR; containing 1x Taq buffer, 1 units of Taq polymerase, 2 mM $MgCl_2$, 200 µm of each dNTPs and 1 µM of each of the forward and reverse primers (5' -GTGCCTTGGGTGAGAGGTTA-3'/5'-CACTCTGCCGTTCTCTTTCC-3'). The thermocycling was conducted for 35 cycles with annealing temperature of 65°C.

The amplicons were digested with XbaI enzyme as per manufacturer's instructions and the digested pattern was observed after separation on 4% agarose gel electrophoresis. The presence of the XbaI polymorphism within the intron 7 of the mucin4 gene corresponds to the presence of F4ac/ab receptor. Thus a single band of 367 bp is indicative of homozygous (RR) resistant piglets, 3 bands of 367 bp, 216 bp and 151 bp represent heterozygous (RS) susceptible piglets, while two bands of 216 bp and 151 bp represents homozygous (SS) susceptible piglets.

Such twenty-one seronegative, and heterozygous (RS) susceptible piglets were selected for further *in vivo* experiment.

Challenge strain and bacterial inoculum

The pathogenic isolate, *E. coli* strain GIS 26 (O149:K91:F4ac, LT^+, STa^+, STb^+)(Cox and Houvenaghel, 1993) was used in this experiment. For convenience of

screening the pathogen post infection, streptomycin resistance of the strain was raised in step wise manner unto 1 mg/ml of medium by progressive screening for bacterial colonies that grew on higher dose of streptomycin. Prior to infection presence of all the toxins in this streptomycin (1 mg/ml) resistant strain was confirmed by PCR. The expression of F4 fimbriae and its potential to attach to F4R was confirmed by an *in vitro* adhesion test, and this strain was named GIS26R^{strep}. One day before challenge, the inoculum was prepared with GIS26R^{strep} as described previously (Snoeck *et al.*, 2003), briefly single colony was inoculated in Tryptone Soya Broth (Oxoid, Basingstoke, UK) and cultured overnight at 37°C and 85 rpm, bacteria were collected by centrifugation (2000g, 35 minutes at 4°C) and resuspended in PBS (pH 7.4) to an optical density of 1 at wavelength of 660 nm, where OD of 1 corresponds to 10^9 bacteria/ml (Snoeck *et al.*, 2003).

Experimental set up and feeding regimen

All the experimental procedures involving piglets and their maintenance was done in accordance with the Belgian legislation for animal welfare and were approved by the animal care and ethics committee of the Ghent University, Belgium.

Hundred suckling piglets raised in conventional Belgian porcine farm (Belgian Landrace x English Landrace) were screened, of which 21 piglets from 4 litters, seronegative for anti-F4$^+$ETEC antibody and heterozygous for F4R receptor gene (genotype-RS) were selected for the feed-challenge experiment. The piglets were challenged twice the first day of challenge was accounted as day 0. On the day -14, (at about 3 weeks of age), the piglets were weaned and brought to the laboratory stables, where for first 8 days (day -14 to -7) they were housed together in one pen at 24±2^{0}C, with water and flax-feed containing 2 gram colistin per kg of feed was available ad libitum.

On the day -6 all the piglets were weighed, given ear tag number from 1 to 21 and separated into 4 pens respectively for each of the 4 experimental feed-treatment groups. Within these pens water was provided ad libitum while the experimental feed was regulated, daily 350 grams of respective feed was given

per pig in the common feeding units installed in each pen. The negative control group (NC) included 7 piglets (piglet number 1- 7) and received feed with milled wild type Arabidopsis seed. Next 7 piglets, no. 8 to 14 constituted the 'VHH-IgG-80' group receiving the VHH-IgG-80 feed, average daily consumption of which would lead to a dose of ~80 mg of VHH-IgG per pig per day. Piglets 15, 16 and 17 comprised the VHH-IgG-20 group and received low dose IgG treatment in feed VHH-IgG-20, daily dietary allowance of this feed formulation would provide ~20 mg of VHH-IgG per pig per day. The remaining 4 piglets (no. 18 to 21) received VHH-IgA-20 feed bearing an cocktail of VHH-IgA antibodies (mIgA, dIgA and sSIgA), also at a concentration to enable ~20 mg dose per pig per day. However after moving the piglets, some distress and loose faeces was noticed in a few piglets, most likely due to moving stress. Hence as precaution colistin was administered orally on day -3 and on day -2 flax feed was given. On day -1, piglets showed normal signs of healthy behaviour and the experimental feed regime was started fresh again. The experimental feed with milled Arabidopsis seeds was fed from the day -1 until day 4 of the experiment. After day 4 the feed in each pen was switched to flax-feed, which was then provided ad libitum until day 11 when the piglets were euthanized, secundum artem, by injecting an overdose of Nembutal (60 mg/kg body weight).

Challenge: To reduce the bacterial gut flora, on day -6 and -5 the piglets were orally given a broad spectrum antibiotic mix of 1 ml florfenicol (Nuflor®) and 2.5 mg/kg body weight of Baytril (enrofloxacin) in 5 ml of PBS, and further 150,000 U/kg of colistin was administered orally on day -3. After the antibiotics cleared from the piglet system, on the day 0 and day 1 the piglets were challenged via intra-gastric inoculation of 10^{10} bacterial particles in accordance to the protocol described previously (Cox *et al.*, 1991) with the exception that the piglets were not fasted or deprived of water both before and after the challenge. Briefly, the piglets were sedated with 2 ml azaperone (Stressnill® Janssen Animal Health), the gastric pH was neutralised with 60 ml of $NaHCO_3$ (1.4% w/v in distilled water) administered via orogastric intubation, 15-30 minutes later via similar intubation 10 ml of challenge bacterial suspension in PBS (10^9 bacteria/ml) was inoculated. During the course of the experiment the weight gain was evaluated by measuring the body weight on day -6, -3, 1, 3, 5, 7 and 11. Blood samples were

taken from the jugular vein on the day -3, 4, and day 11; while faecal sample was taken from the anus (or through rectum) on the day -3, day 1 to 8 and day 11. After euthanizing, the piglets were dissected; the content from ileum and caecum was taken and processed similarly as faeces sample. Also ~20 cm long segment of the jejunum was excised washed with sufficient PBS followed by Krebs-Henseliet buffer and fixed by incubation for 60 minutes in Krebs-Henseliet buffer with formaldehyde (1% v/v). The villus enterocytes were scrapped from this segment and used to reconfirm the phenotypic expression of the F4R receptors via adhesion assay.

Determination of $F4^+$ETEC shedding in faeces

The faeces collected were kept on ice, and processed immediately; a cold chain was maintained until platting of the faecal dilution. Firstly, a 10% (w/v) faecal suspension was made in PBS, and serially diluted from 10% stock to $1x10^{-5}$, 100 μl of each dilution was plated with glass beads on blood-agar plates (OXIOD) with 1 mg/ml streptomycin and 20 μg/ml of tetracycline as selection antibiotics and 10% v/v defibrinated sheep blood (Bio-trading). The inoculated plates were incubated at 37°C overnight, and the haemolytic $F4^+$ETEC bacteria were counted and the bacterial count was dually confirmed via colony blotting — where a circular PVDF membrane is activated in methanol and then placed on the colonies in the Petri plates for 2 hours at room temperature, the blots were then blocked in blocking solution (5% skimmed milk in PBS) overnight at 4°C; subsequently the blots were washed 3 times in PBS and incubated in a 3% skimmed milk in PBS bath with anti-F4-HRP (monoclonal antibody isolated in Lab of Immunology, UGent) for 1 hour at room temperature. The blots were washed again 3 times with PBS and developed with AEC (3-amino-9-ethylcarbazole) (Sigma) substrate according to the manufacturers instructions. After 15 minutes of incubation with the substrate the membranes were washed with water and the precipitated coloured dots specifically representing $F4^+$ETEC were counted.

Determining titer of piglet anti-F4$^+$ETEC antibodies in serum after challenge

To determine the titer of anti-F4+ETEC antibodies in the serum, an ELISA setup as described above was used. Serum was processed as mentioned from the blood collected on— day of screening, day -3, 4 and 11. All the samples were loaded in duplicate, together with positive and negative reference serum. The OD of the colorimetric product developed at the end of the enzyme-substrate reaction was measured at 405 nm and the rise in serum anti-F4-IgG, anti-F4-IgM, anti-F4-IgA, and total Ig levels was evaluated (Verdonck *et al.*, 2004b) using the detection antibody anti-pig IgG (Bethyl a100-104a), anti-pig IgM (Bethyl A100-100A) anti-pig IgA (Bethyl A100-102A-13) and anti-pig (total) (Dako z0139) antibodies respectively. The seroconversion was compared between all the pigs and within the 4 groups.

Statistical analysis

The results over time were analyzed using a General Linear Model (repeated measures analysis, Statistica 10.0, Statsoft, Tulsa, USA), with treatment as fixed factor.

Supplementary data:

Supplementary Table 6.ST1: Daily log of bacterial shedding and F4 receptor status for each piglet.

piglet no.		F4⁺ETEC shedding post challenge (log_{10} bacteria per gram of faeces)									F4R status (average F4⁺ETEC/ 250µm of villous surface)
		day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	day 11	
1	Negative control	4.76	4.11	x	x	3.30	x	x	x	x	25.25
2		4.34	3.60	2.00	x	x	x	x	x	x	40.00
3		4.04	3.60	3.48	2.78	3.30	x	x	x	x	7.00
4		6.84	6.56	4.76	4.08	3.79	3.60	2.48	x	x	40.25
5		6.72	6.54	6.99	7.77	5.08	5.09	2.90	x	x	16.25
6		5.40	5.58	3.30	2.93	3.30	2.00	2.70	2.30	x	24.75
7		4.99	4.35	4.08	2.30	2.00	x	x	x	x	18.50
8	VHH-IgG-80	4.36	n.d.	5.99	3.71	x	x	x	x	x	22.00
9		5.39	4.81	3.00	3.22	4.30	4.63	3.48	3.41	x	34.00
10		7.49	6.40	4.69	4.13	5.78	4.15	2.70	x	x	37.25
11		n.d.	6.76	5.08	3.71	3.60	3.60	2.30	2.00	x	22.50
12		8.97	7.50	5.05	5.10	5.10	5.52	3.00	3.08	x	23.75
13		5.90	3.95	3.00	x	x	x	x	x	x	31.00
14		6.94	6.64	3.00	x	x	3.30	x	x	3.08	19.50
15	VHH-IgG-20	7.58	5.62	x	x	x	x	x	x	x	7.50
16		6.22	6.13	4.51	x	x	x	x	x	x	36.25
17		3.00	x	4.00	x	x	x	x	x	x	5.75
18	VHH-IgA-20	6.48	5.70	3.00	x	x	x	x	x	x	23.00
19		4.31	3.85	x	x	x	x	x	x	x	30.00
20		6.83	4.27	3.00	x	x	x	x	x	x	29.75
21		7.35	5.31	x	x	x	2.00	2.00	x	x	45.75

The log_{10} bacterial count per gram of faeces detected has been tabulated, 'x' indicated no bacteria detected where the detection limit was 100 bacteria per gram of faeces. No faecal sample was obtained from piglet no. 8 and 11 on day 2 and 1 respectively, which is denoted as 'n.d.' in the table. The number of F4⁺ETEC bacteria attached to the villous surface is a direct indicative of the F4 receptor (F4Rac) phenotypic expression. F4⁺ETEC more than 30 per 250 µm of villous surface is indicative of strong positive, 5 to 30 bacteria is regarded as moderately positive while less than 5 is classified as F4R negative piglet. The vertical line indicated the time when prophylactic-treatment feed was changed to flax-feed with no antibody.

Supplementary Table 6.ST2: Weight chart of the piglets

		Piglet weight in kg measured on the following days							piglet weight gain in kg	
piglets		**day -6**	**day -3**	**day 1**	**day 3**	**day 5**	**day 7**	**day 11**	from day -3 to day 5	from day 5 to day 11
1	control	7,1	7,8	7,4	8,2	9,3	10,0	10,5	1,5	1,2
2		7,8	8,3	7,9	9,1	9,5	10,4	11,1	1,2	1,6
3		7,9	8,7	7,8	8,1	9,9	11,1	11,5	1,2	1,6
4		6,4	6,6	5,6	6,8	7,8	9,1	9,0	1,2	1,2
5		9,9	8,4	8,3	9,8	10,1	11,5	12,3	1,7	2,2
6		7,8	10,1	9,6	11,1	12,9	13,6	14,2	2,8	1,3
7		7,8	8,1	7,3	8,8	9,6	10,8	11,5	1,5	1,9
8	VHH-IgG-80	6,9	5,4	5,6	6,5	7,2	7,4	9,0	1,8	1,8
9		6,5	6,9	7,5	7,0	7,4	7,8	9,0	0,5	1,6
10		6,7	6,9	8,7	9,0	9,3	9,8	12,5	2,4	3,2
11		6,4	6,6	6,9	7,3	8,0	8,1	10,0	1,4	2
12		7,7	8,6	8,7	9,6	10,2	10,1	12,1	1,6	1,9
13		7,9	8,1	10,5	10,8	11,4	12,0	14,3	3,3	2,9
14		8,0	8,7	9,4	9,6	11,4	11,3	14,4	2,7	3
15	VHH-IgG-20	6,4	6,7	7,7	8,0	8,7	10,1	11,9	2	3,2
16		5,0	5,1	5,7	6,2	6,9	7,8	9,7	1,8	2,8
17		7,4	7,8	8,3	8,9	10,4	11,3	13,6	2,6	3,2
18	VHH-IgA-20	6,1	6,5	7,4	8,1	8,2	9,1	11,0	1,7	2,8
19		6,5	6,8	6,4	7,7	8,1	8,9	10,1	1,3	2
20		8,9	9,1	10,1	10,6	11,3	12,6	15,5	2,2	4,2
21		n.w.	8,2	9,0	9,5	10,1	11,3	13,0	1,9	2,9

(Here n.w. stands for not weighed)

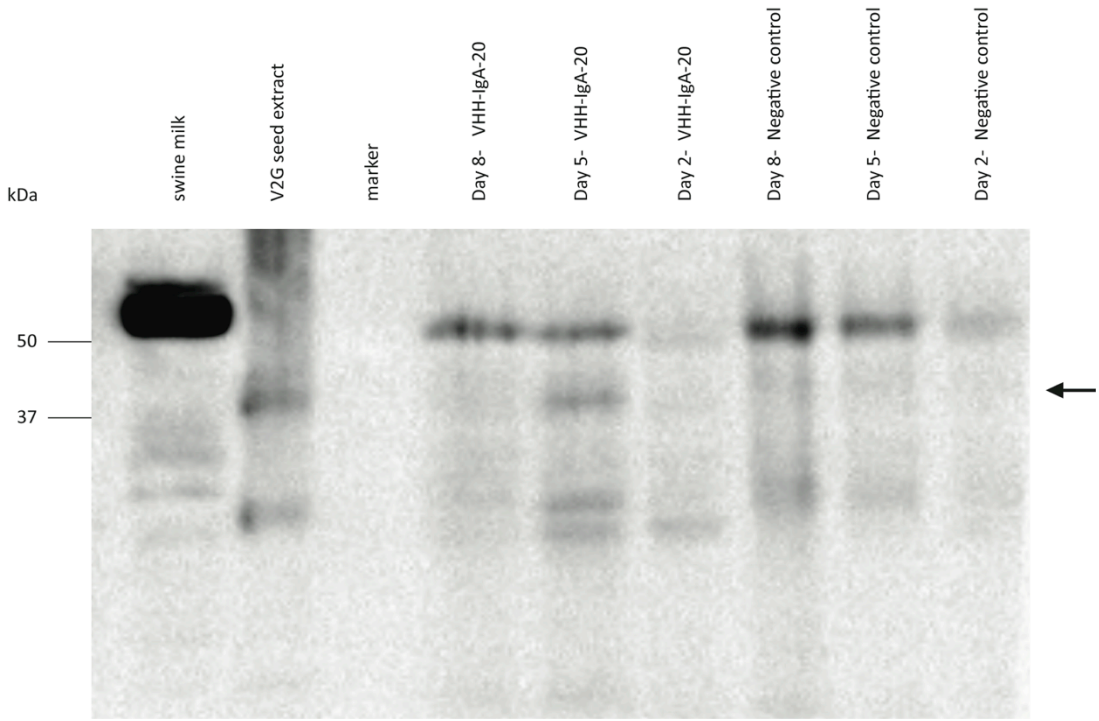


Figure 6.S1: Detection of plant made VHH-IgA in piglet faeces. To detect the presence of plant made antibodies in faeces, a 200 µl pool of faecal suspension was made from all the 4 piglets of the VHH-IgA-20 group by mixing 50 µl of 5% faeces suspension of each; such pools were made from the faeces collected on day 2, 5 and 8. Similar pools were also made for 7 negative control piglets. To enrich the IgA antibodies in this pooled suspension, batch purification was performed with SSL7/agarose resin and 10 µl of the elution was immunoblotted under reducing condition (12% SDS-PAGE). Detection with anti-pig IgA polyclonal antibody showed the presence of plant made VHH-IgA (~ 37 kDa, indicated with arrow) only in the VHH-IgA-20 group on day 5. All the other pools of VHH-IgA-20 and negative control did not bear this (lanes annotated in the figure); as control for porcine IgA swine milk was used, while seed extract V2A was used for control for plant made VHH-IgA. The heavy and light chains of porcine IgA in faeces can be seen at ~ 55kDa and 25kDa.

Chapter 7

General conclusions, discussion and perspectives

In seed production of antibodies for oral passive immunisation—Lessons learnt for the road ahead

In a nut shell

Passive immunisation strategies have an unrealised potential for prevention and treatment of several human and animal infections. The present high cost of the recombinant antibody production limits the wide spectrum use of passive immunisation. Several non-conventional expression systems are being utilised to attain high production level. Since production levels are inversely proportional to the cost of final therapeutic product it is hoped that in the future passive immunisation would be affordable to include in the basic human health care system and also used for animal health. Amongst these non-conventional methods plants are emerging as a promising platform to achieve the low cost antibody production. In particular expression in seeds is an exciting opportunity for oral in feed administration of antibodies to farm animals. In this Ph.D. thesis we deliver the proof of concept of such prophylaxis, in preventing $F4^+$ETEC infection in piglets, by production of novel antibodies in the seeds of *Arabidopsis thaliana*.

Broadly, the seed made antibodies were robust and remained active *en route* gastric transit. The VHH-IgA-20 feed containing the cocktail of VHH-IgA antibody formats at a concentration that would enable antibody dose of 20 mg/pig/day, inhibited the colonization of the challenge strain leading to speedy bacterial clearance from the piglet system. This group also had the highest weight gain towards the end of the experiment, compared to the control and the VHH-IgG antibody receiving groups. The VHH-IgG antibody bearing experimental feeds evaluated in this study failed to demonstrate similar protection. In fact the VHH-IgG-80 treatment group (dose of ~80 mg VHH-IgG antibody per pig per day) showed prolonged shedding of bacteria compared to the negative control group, which received wild type Arabidopsis seeds. Also as compared to the negative control a higher and faster seroconversion was seen in the piglets of the VHH-IgG-80 group. The porcine IgG3 Fc of the VHH-IgG perhaps interacts with the porcine neonatal Fc receptor present on gut epithelial cells enabling prompt trans-epithelial transport of the antigen resulting in earlier triggering of immune system and higher seroconversion. Interaction of $F4^+$ETEC bacteria to VHH-IgG bound to the epithelial FcRn receptor might have also influenced the

colonisation; it would be interesting to evaluate this hypothesis in the future. And if this turns out to be correct then as an alternative other IgG isotopes, which have lower affinity for FcRn can be experimented as fusion partners for VHH.

The ability to protect against $F4^+$ETEC challenge and furthermore curtailing bacterial shedding, present the VHH-IgA prophylaxis with higher merits as candidate antibody format for evaluation in a field trial. Transformation of the VHH-IgA constructs in seed crops like soybean would enable bulk production of VHH-IgA bearing feed for a prospective large-scale field trial.

The lessons learnt

We learnt that VHH-IgA based mIgA, dIgA and sSIgA formats might be suitable antibody designs for oral in seed based prophylaxis. However, it needs to be determined if all the three formats are necessary or any one of the formats would suffice for same efficacy. At the moment only a fraction of the VHH-IgA actually formed the assembled molecule. In case of the dIgA and sSIgA, the results from the molecular characterisation (Chapter 5) hint that, for attaining higher accumulation of assembled functional molecule, the ratio of accumulation of the constituent elements needs to be fine-tuned. Also the aberrant glycosylation and the proteolysis of VHH-IgA chain *in situ* might be responsible for inefficient assembly. Two basic strategies could be adopted to boost the recovery of functional dIgA and sSIgA; either by fine tuning the regulatory elements or by conventional crossing and breeding techniques.

The β-phaseolin promoter has usually led to high accumulation in Arabidopsis seeds in the past (De Jaeger *et al.*, 2002; Van Droogenbroeck *et al.*, 2007). The highest accumulation achieved was for a murine single chain variable fragment (ScFv) which reached up to 36.5% of total soluble protein (TSP) (De Jaeger *et al.*, 2002) while in another study which expressed ScFv-Fc, three such antibodies expressed from 7% to 12% of TSP (Loos *et al.*, 2011b; Van Droogenbroeck *et al.*, 2007). Theoretically, the proportion of J chain and SC required is ideally 4 times lower than the VHH-IgA chains for sSIgA production. One could attempt swapping the β-phaseolin promoter with another promoter like USP or CaMV 35S the latter is a constitutive promoter and has been reported to lead to lower expression in seeds; thus the relative expression of the sSIgA elements could be

harmonised (Boothe *et al.*, 2010; De Wilde, 2012). Alternatively other regulatory elements at the 5' and 3' end could be fine tuned, as shown in case of the transient expression vector pEAQ, where the modified 5'UTR leads high expression of more than 10 to 20% of TSP for most heterologous protein (Sainsbury and Lomonossoff, 2008; Sainsbury *et al.*, 2009; Thuenemann and Lomonossoff, 2010).

Another strategy would be to cross the plant lines expressing high levels of VHH-IgA with a transformant that express lower levels of J chain and SC. Such candidate J chain + SC expressing plant lines could be identified from our pool of transformants. On the same principles the ratio of J chain to VHH-IgA can be adjusted for higher dIgA production.

In chapter 4, we described the production of VHH-IgG divalent anti-F4$^+$ETEC antibodies, which were functional *in vitro*, but did not prevent the bacteria from establishing the infection at a dose of 80 mg/piglet per day. While, the protective effect in case of lower dose of VHH-IgG at 20 mg/piglet per day could not be evaluated since the piglets did not bear sufficient receptor necessary for susceptibility to F4$^+$ETEC bacteria. Hence it is difficult to conclude if the 20 mg dose would have been protective; if so, it would also suggest that a higher dose of antibody is detrimental and ineffective. Without evidence, this statement remains speculative in nature, and would need further evaluation. Once the effect of VHH-IgG at 20 mg daily dose per piglet is evaluated it can then be compared with the 20 mg dose of VHH-IgA oligomeric cocktail in treatment feed per piglet per day. For re-evaluation of these questions and to take this concept close to valorisation, it is ideal to transfer these constructs in crop seeds. Along with production in crop seed, high accumulation will be necessary to reap any benefits.

Attaining high accumulation

With the aim to achieve highest accumulation of the anti-F4$^+$ETEC antibodies we chose the β-phaseolin promoter and the 3' arceline terminator bearing regulatory sequence, the combination of these gene regulatory elements have led to a very high accumulation of antibodies in Arabidopsis in the past. In chapter 4,

on expression of 4 different anti-FaeG VHHs (V1, V2, V3 and V4) fused to porcine IgG3 Fc under the control of the β-phaseolin promoter led to differential accumulation of these fusion antibodies. The VHH-IgG fusion antibodies V3G and V4G reached accumulation of 2% of TSP, V1G expressed up to 10% of TSP; while V2G reaching the maximum expression in this thesis of 15% of Arabidopsis seed proteins. From the discrepancy of their accumulation we speculated that the VHH being the only variable segment, perhaps influence the accumulation of VHH-IgG. We also proposed the substitution of complement determining regions (CDR) from the low expressing V3G into the antibody V2G to evaluate if this would lead to higher expression, and by doing so if unique framework regions for high accumulation of VHH-IgG can be determined. It would have to be evaluated in time if such loop-swapped chimera VHH would have the same binding affinity.

In chapter 5, when the same 4 VHHs were grafted on to a different porcine Fc of IgA^b, we no longer observed the discrepancy in accumulation of the 4 VHH-IgAs. All of the VHH-IgA accumulated to the same level, which was much lower (~1% of TSP) than VHH-IgG fusion. This VHH-IgA fusion molecule was also less stable in Arabidopsis seeds (*in situ*) and was prone to degradation and aberrant glycosylation.

In another project in our group we fused the same porcine Fc of IgA^b and IgG3 to another VHH called VHH7 (against the human prostate specific antigen). The maximum expression of the VHH7-IgG was up to 2% of TSP and the VHH7-IgA accumulated to only 0.8% of TSP, while when fused it to the Fc of murine IgG3 the accumulation increased up to 7% of TSP. The results suggest that the stability presented by the Fc is important for high accumulation of VHH-Fc. And further by engineering the VHH framework regions the accumulation could perhaps be boosted.

In addition the aberrant glycosylation of the protein might also influence the accumulation. In our initial analysis of the VHH-IgA glycans it seems that the Man7 residues are the most abundant form of glycans on VHH-IgA. Man7 occurs in three isomeric forms Man7.1, Man 7.2 and Man 7.7. Of these Man 7.1 has been suggested to serve as a glycan tag for miss folded proteins to be targeted for the

ER-associated degradation pathway (Clerc *et al.*, 2009). Further analysis of the isoform of the high Man7 residue will shed more light on these questions.

Classical breeding techniques in increasing accumulation

Classical breeding techniques have been used since ages to increase the yield of crops or to obtain better vigour. Such breeding techniques have also proved to increase the accumulation of recombinant antibodies made in plants (Hood *et al.*, 2002). A transgenic maize line producing a recombinant industrial enzyme resulted in 70-fold increase in accumulation on crossing it with an elite germplasm, and in case of avidin producing maize lines this method resulted in increase of up to 150-fold in accumulation (Hood *et al.*, 2002). Recently the seed yield parameters for Arabidopsis mutant and transgenic lines has been analysed in detail (Van Daele *et al.*, 2012). Candidate plants from this collection can be crossed with antibody producing lines to evaluate if this crossing leads to higher antibody accumulation in F1 progeny. This technique would be very beneficial for attaining higher accumulation in crop seeds. The high yielding varieties and know-how for classical breeding techniques already exist for most of the crops like soybean, pea, bean etc.

V2G X V3G – production of oligomer cocktail in one line

Apart from the proposed strategies to swap the CDRs of V3G with V2G, one could also try crossing the high expressing line (e.g. V2G) with low expressing line (e.g. V3G), it will be interesting to evaluate what happens to overall accumulation then. In conjunction, this method will enable production of oligomer cocktail in one line. In principle a V2G x V3G line would produced bispecific VHH-IgG antibody with V2 and V3 VHH domains (V2,V3-G) along with homodimers with two V2 or V3 domains (V2G and V3G).

Such strategy of filial crossing can be incorporated for bringing in more antibody elements in one plant line; these antibodies could be against different epitopes of the same pathogen, or different pathogens. For instance along with anti-F4$^+$ETEC antibodies, similar antibodies against other diarrhoea causing bacteria with F18 fimbriae bearing ETEC could be isolated, produced in plants and crossed together. Thus aiming to make a disease specific seed based therapeutic.

Weather a bispecific antibody produced in seeds would have added benefits than merely mixing two antibodies producing feed, in terms of efficiency against the antigen would need to be investigated.

These tips discussed above could be effectively utilised in attaining high expression in crop seeds. However, the later discussed crossing technique might be more suitable for crops, since transformation of crops like pea and soybean is a lengthy and difficult process.

From Arabidopsis to crop seeds

Arabidopsis thaliana – a nuisance weed to most but has been a boon to the plant research community. However for molecular farming, particularly for in seed expression, crops like– pea, soybean, safflower, maize and rice are preferred. An important reason for this preference is the seed yield per unit area. Additionally, the agriculture infrastructure for processing these crops are already available. In comparison Arabidopsis is a strong contrast when it comes to scalability and agronomics, the size of the seeds is less than 400 micron, the seed yield per plant is about 100-200 mg, i.e. about 30 g - 60 g per square meter and bears a very high cost of scale up (> $25 US per gram[20]). Thus it is not suitable for large-scale seed based oral passive immunisation of animals. But on the brighter side the transformation of Arabidopsis is very easy and required merely dipping of flowers in a diluted *Agrobacterium* culture, thus large number of primary transformants can be screened and characterised. In conjunction to this, the model plant can be conveniently grown in green houses or growth chambers and has a short life span. These merits enabled the swift development of customised anti-F4$^+$ETEC antibodies that were evaluate *in vivo* in the disease model. By using Arabidopsis platform we could select and prioritise the ideal antibody candidates for further expression in crops seeds.

Unlike Arabidopsis, transformation of crops like soybean and pea is very tedious, requiring skilled experts and is time consuming. From our expression results in Arabidopsis seeds, we chose 3 antibody constructs for evaluation of expression in the seeds of pea and soybean; these were— 2 VHH-IgG antibodies, the high

[20] Quotation received from a Lehle seed company, personal communication

expressing V2G, low expressing V3G and the VHH-IgA, V2A. These three antibody constructs were transformed in pea by *Agrobacterium*-mediated transformation of embryo explants (Figure 7.1) in accordance to the protocol described (Polowick *et al.*, 2000).

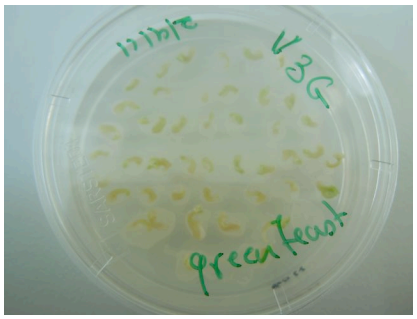


Figure 7.1: Transformation of V3G in pea, the image shows the co-cultivation step in the pea transformation protocol. Thin slices of embryo explants dissected from imbibed seeds were co-cultivated with *Agrobacterium* strain bearing V3G within the pPhasGW expression cassette. Transformation of pea was performed in the lab of Prof. Inge Broer at the University of Rostock.

The soybean was transformed by *Agrobacterium*-mediated transformation using half seed (cotyledon node) as explant (Paz *et al.*, 2006) (by the plant transformation service of the Iowa State University). The seeds from the primary transformants of both these plant species are expected in August 2012, after which the expression and efficacy of pea and soybean produced antibody would be evaluated.

Piglet feed is either available as milled flour or in the form of pellets, the preference differs in accordance to the available animal husbandry infrastructure. To make a product for a broader market the antibody producing pea and soybean will be tested for antibody survival and stability on extrusion to make feed pellets. These stability experiments are planned first quarter of 2013.

Why pea and soybean?

Both pea and soybean are routinely used in piglet feed as source of protein. Recently the "Feasibility of pea as an expression system for pharmaceuticals" has been excellently reviewed (Mikschofsky and Broer, 2011). Pea contains about 40% protein, thus VHH-Fc antibody expression of up to 2% of TSP would correspond to about 8 mg VHH-Fc per gram fresh weight. This proportion is higher than in Arabidopsis seeds, meaning that if the pPhas expression cassette leads to equally high accumulation of antibody as seen in Arabidopsis seed, then the final inclusion rate of pea in piglet feed would be less. This would be advantageous for two reasons- nutrition and regulation. Some legumes like pea

and soybean do have anti-nutritive factors, due to which they are sparingly used in feed formulation. However up to 30% inclusion is permissible. Maintaining low inclusion rate might be more important for certification/regulation of feed. Further, pea can be grown in temperate countries like most of Europe, and soybean is a crop grown in abundance in lower latitudes.

Some of the major issues and challenges in production of antibodies in seeds is the GM regulation. Globally the policies related to GMO are different in each country. We realize that growing GM-crops producing antibodies in seed will be very difficult/impossible within the E.U. in the immediate future, but growth of GM soybean for feed should be no problem in other parts of the world, and import of GM-soybean in Europe has become common practice (90% of imported soya for feed in Belgium is labeled as GM-derived). Soybean takes about 4 months to maturity; the cost of producing soybean is less than crops like peas, beans, peanuts and most cereals. Moreover, soybean is a self-fertilizing crop in which only a very low percentage of cross pollination takes place (approximately 1%, and is rare at distance more than 6 m), so outcrossing from one field to an adjacent field is estimated to be very unlikely but cannot be disregarded to be absolutely zero (Abud *et al.*, 2007). Contamination of other crops for human consumption as a result of volunteers[21] is also estimated to be very unlikely as seeds of soybean do not exhibit any dormancy. Other forms of contamination can result from the use of machinery that has not been cleaned properly after use in antibody producing crop. Cleaning of machinery however is rather simple activity, but requires strict compliance. Danger for contamination can be very seriously diminished when the antibody producing soybean would be cultivated in an area where no, or very limited other soybeans are grown (such regions do exist, for instance India, where the climate is favorable but soybean plantation is pocketed). Additionally immediate grinding of the soybean flour near harvesting facility would limit the very slim chances of contamination during transport. The long term stability of such seed produced antibody would have to be determined in grinded seed flour would have to be determined on the lines of the stability

[21] Definition: Volunteers- inadvertently growing plants resulting from dispersal of seeds by biotic or abiotic means

experiment performed with Arabidopsis seed (Chapter 4).

Alternatively, soybean can also be grown in greenhouses. In fact it has been reported that exploitation of the photoperiod in green house can lead to 10 times increase in seed yield (Vianna *et al.*, 2011). Can this be a solution to looming GM risk related issues? Well, a detail evaluation might help to investigate this possibility for molecular farming.

Prediction for the future

The possibility to prevent ETEC related PDW and related piglet mortality would prevent annual losses and provide a huge economic boost to the porcine rearing industry. In a developed country like Belgium, 2% of the weaning piglets die, which accounts for ~300,000 deaths per year. At the rate of 30 euro per piglet, the economic loss is EUR 9 million per annum. Economic loss due to piglet mortality in developing countries can bear direct detrimental consequence on the basic livelihood of the farmer. Even the surviving piglets have reduced weight gain, which adds to the economic burden; overall every year Belgian porcine industry faces a loss of EUR 15 million. This economic loss is mainly due to the ETEC induced PWD. There is a huge market demand for an effective product; especially since the products currently available in market fail to effectively prevent ETEC related PWD.

The in seed made antibody strategy not only ensures a specific anti-ETEC product to meet this demand, but it aims at achieving this at a cost effective rate. The production cost of soybean containing F4-specific antibodies will be influenced by various market parameters, and will be a subject of a follow-up study. Deregulation and obtaining the precise certification is one of the foreseen expenses in the road to valorization. Usually, apart from production cost the expenses incurred during valorization and marketing phase, largely influence the final cost price of therapeutics. We estimate the production cost of adding antibody-producing soybean at a ratio of 2% to the starters feed would be approximately less than EUR 20 per ton of feed. A ton of basic starter feed cost about EUR 400 in the market, and feed with 4% plasma protein, which is sold as ETEC prophylactic feed, whose efficacy is questionable, cost EUR 560 per ton. Comparing which, in terms of cost, soybean-containing feed would have a margin

of EUR 140. It would be needed to evaluate in future if this margin would compensate for the liable regulatory expenses, marketing costs, research and development depreciation etc. and recover a profit on the final sale.

Summary

Summary

Post-weaning diarrhoea caused by F4 fimbriae bearing enterotoxigenic *Escherichia coli* (F4⁺ETEC) is a common disease in piglet rearing industry. Such F4⁺ETEC infections routinely cause morbidity leading to reduced weight gain and in acute cases (~2%) lead to mortality[22] (Fairbrother *et al.*, 2005). This consequentially results in heavy economic losses to the global porcine industry, seriously affecting the profit margin of the large scale porcine farms in the western hemisphere while in developing countries it can affect the daily livelihood of a subsistence farmer (Amezcua *et al.*, 2002; Hong *et al.*, 2006). Prophylactic use of antibiotics has been hugely successful in the past, however the risk of introducing resistance strains has led to the prohibition of prophylactic antibiotic application. This crisis has led to an urgent demand for a successful treatment (Adjiri-Awere and Van Lunen, 2005).

Passive immunisation has been suggested as an alternative to antibiotic use in animals and human bacterial infections (Berghman *et al.*, 2005; Oleksiewicz *et al.*, 2012). In different piglet-challenge experiments, the prophylactic administration of either anti-F4⁺ETEC monoclonal antibodies, or immunised egg protein, or animal plasma as source of antibodies has been successful in preventing F4⁺ETEC infection (Marquardt *et al.*, 1999; Niewold *et al.*, 2007; Yokoyama *et al.*, 1992). However it has been difficult to implement these techniques outside the laboratory, since immunised hen egg antibodies are expensive and there are concerns about the safety and efficacy of animal blood derived plasma, owing to which use of animal plasma in feed is strongly discouraged.

In this research project, we envisaged production anti-F4⁺ETEC antibodies in plant seeds that can be incorporated into the feed of weaning piglets. To this end, as a proof of concept, we designed a novel robust antibody by fusing the antigen binding domains of camelid antibodies (VHH) with the fragment crystallisable (Fc) of porcine immunoglobulin IgG3 (proteolytically stable). Four anti-F4⁺ETEC VHHs were selected via panning from a lymphocytic cDNA library derived from a

[22] From the archives of Dierengezondheidszorg Vlaandern (Animal health care, Flanders) http://www.dgz.be/

lama immunized with purified major adhesion molecule- FaeGac, which makes the F4 fimbrial shaft. The VHH-IgG fusion DNA coding sequences were introduced into a seed specific expression cassette and transformed in Arabidopsis. Transformants with each of these VHH-IgG antibodies were screened for the highest antibody producing seed stocks. Of these VHH-IgG antibody, the one named V2G accumulated to very high levels of about 3% of seed weight (i.e. ~30 gram antibody per kg of seeds); while another named V1G accumulated to ~2% of seed weight, while the remaining two named V3G and V4G each accumulated to about 0.4% of seed weight. All the 4 VHH-IgG antibodies were functional in an ELISA assay and agglutinated the bacteria. Thus proving that **functional VHH-IgG antibodies can be produced in high amounts in seeds** of transgenic plants (Chapter 4).

Further based on the same VHH-Fc fusion strategy we **developed a novel format of secretory IgA (SIgA)** antibodies. Secretory IgA antibodies are the predominant class of antibodies at the mucosal surface and they are involved in establishing a first line of defense (Corthésy, 2003). Role of mucosal immunity is likewise also suggested to be essential in preventing the pathogenesis of $F4^+$ETEC in piglets (Verdonck *et al.*, 2004b). By grafting the 4 isolated VHHs onto the Fc fragment of porcine IgA and co-expressing these fusions with porcine joining chain (J chain) and porcine secretory component (SC) in seeds of Arabidopsis plants, we demonstrated that three different and unique functional formats of IgA can be produced– monomeric, dimeric and secretory IgA like molecules designated as **simplified secretory IgA (sSIgA)**. The accumulation of the assembled molecules depended on the expression of each element, mainly the J chain and SC; while the expression of the 4 VHH-IgA antibodies was roughly equivalent in all seed stocks analyzed. The overall production levels of these functional assembled sSIgA molecules was estimated to amount to about 0.2% of seed weight (Chapter 5).

All the antibody producing seed extracts (each of the 4 VHH-IgG and the three formats of the VHH-IgA i.e. monomeric, dimeric and sSIgA, bearing the same 4 VHH antigen binding domains) specifically **agglutinated the $F4^+$ETEC bacteria and inhibited the interaction with gut villous enterocytes *in vitro***. Further

the protective efficacy of these antibodies was evaluated in an animal challenge experiment. Milled Arabidopsis seeds with and without antibodies were added to the feed of weaned piglets prior to challenge with high dose of F4$^+$ETEC bacteria. Throughout the experiment, the daily shedding of F4$^+$ETEC bacteria was determined, the seroconversion of F4 specific serum immunoglobulin (IgM, IgG and IgA) were analyzed and the piglet weight were recorded. Analysis of which showed that a prophylactic feed formulation with an oligomeric cocktail of all the IgA based antibody formats at a daily dose of 20 mg/pig (VHH-IgA-20) was protective, while the IgG antibody format was not. The piglets receiving (VHH-IgA-20 feed) showed a swift decline in bacterial titer as compared to negative control piglets that received wild type Arabidopsis seeds in their feed. The results for the piglets receiving VHH-IgG cocktail in their feed were not clear; neither at a dose of 20 mg/pig/day (VHH-IgG-20 group) nor at 80 mg/pig/day (VHH-IgG-80 group). Two of the 3 piglets receiving the 20 mg dose of VHH-IgG feed turned out to be relatively insensitive to the F4$^+$ETEC pathogenesis, as they had very low phenotypic expression of the F4$^+$ETEC receptor (F4R). In the VHH-IgG-80 group, all 7 piglets had moderate phenotypic expression of F4R, and showed prolonged shedding of bacteria, indicating that the VHH-IgG treatment at this dose was not protective. In conclusion, the oligomeric cocktail of **VHH-IgA administered in feed is a promising anti-F4$^+$ETEC therapeutic** for preventing F4$^+$ETEC infections post weaning. For further valorization of VHH-IgA based prophylactic therapy, the technique and the antibody used has been patented (Application no. EP 12182774.5). Also the expression of VHH-IgA will be evaluated in the seeds of pea and soybean as these crops can be bulk produced and are routinely used within the standard piglet feed. Thus this Ph.D. research project has brought the antibody based anti-ETEC prophylaxis one step closer to its translation towards a commercial feed based prophylaxis. We envisage that similar approach could also be used for many other diseases in humans and animals (Chapter 6).

References

References

Abud, S., de Souza, P.I., Vianna, G.R., Leonardecz, E., Moreira, C.T., Faleiro, F.G., Junior, J.N., Monteiro, P.M., Rech, E.L. and Aragao, F.J. (2007) Gene flow from transgenic to nontransgenic soybean plants in the Cerrado region of Brazil. *Genet Mol Res* **6**, 445-452.

Adjiri-Awere, A. and Van Lunen, T.A. (2005) Subtherapeutic use of antibiotics in pork production: Risks and alternatives. *Can J Anim Sci* **85**, 117-130.

Amezcua, R., Friendship, R.M., Dewey, C.E., Gyles, C. and Fairbrother, J.M. (2002) Presentation of postweaning *Escherichia coli* diarrhea in southern Ontario, prevalence of hemolytic *E. coli* serogroups involved, and their antimicrobial resistance patterns. *Can J Vet Res* **66**, 73-78.

Andrabi, R., Kumar, R., Bala, M., Nair, A., Biswas, A., Wig, N., Kumar, P., Pal, R., Sinha, S. and Luthra, K. (2012) Production and characterization of human anti-V3 monoclonal antibodies from the cells of HIV-1 infected Indian donors. *Virol J* **9**, 196.

Baker, K., Qiao, S.-W., Kuo, T., Kobayashi, K., Yoshida, M., Lencer, W. and Blumberg, R. (2009) Immune and non-immune functions of the (not so) neonatal Fc receptor, FcRn. *Semin Immunopathol* **31**, 223-236.

Bakker, D., Willemsen, P.T.J., Simons, L.H., Vanzijderveld, F.G. and Degraaf, F.K. (1992a) Characterization of the antigenic and adhesive properties of FaeG, the major subunit of K88 fimbriae. *Mol Microbiol* **6**, 247-255.

Bakker, D., Willemsen, P.T.J., Willems, R.H., Huisman, T.T., Mooi, F.R., Oudega, B., Stegehuis, F. and Degraaf, F.K. (1992b) Identification of minor fimbrial subunits involved in biosynthesis of K88 fimbriae. *J Bacteriol* **174**, 6350-6358.

Barnhart, M.M., Sauer, F.G., Pinkner, J.S. and Hultgren, S.J. (2003) Chaperone-subunit-usher interactions required for donor strand exchange during bacterial pilus assembly. *J Bacteriol* **185**, 2723-2730.

Basaran, P. and Rodríguez-Cerezo, E. (2008) Plant Molecular Farming: Opportunities and Challenges. *Crit Rev Biotechnol* **28**, 153-172.

Becker, P.M., van der Meulen, J., Jansman, A.J.M. and van Wikselaar, P.G. (2011) *In vitro* inhibition of ETEC K88 adhesion by pea hulls and of LT enterotoxin binding by faba bean hulls. *J Anim Physiol Anim Nutr*, Epub ahead of print.

Bendtsen, J.D., Nielsen, H., von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340**, 783-795.

Berdoz, J., Blanc, C.T., Reinhardt, M., Kraehenbuhl, J.P. and Corthesy, B. (1999) *In vitro* comparison of the antigen-binding and stability properties of the various molecular forms of IgA antibodies assembled and produced in CHO cells. *Proc Natl Acad Sci USA* **96**, 3029-3034.

Berends, E., Scholtmeijer, K., Wösten, H.A.B., Bosch, D. and Lugones, L.G. (2009) The use of mushroom-forming fungi for the production of N-glycosylated therapeutic proteins. *Trends Microbiol* **17**, 439-443.

Berghman, L.R., Abi-Ghanem, D., Waghela, S.D. and Ricke, S.C. (2005) Antibodies: An alternative for antibiotics? *Poult Sci* **84**, 660-666.

Bertschinger, H.U., Nief, V. and Tschape, H. (2000) Active oral immunization of suckling piglets to prevent colonization after weaning by enterotoxigenic *Escherichia coli* with fimbriae F18. *Vet Microbiol* **71**, 255-267.

Better, M., Chang, C.P., Robinson, R.R. and Horwitz, A.H. (1988) Escherichia coli secretion of an active chimeric antibody fragment. *Science* **240**, 1041-1043.

Bhandari, S.K., Nyachoti, C.M. and Krause, D.O. (2009) Raw potato starch in weaned pig diets and its influence on postweaning scours and the molecular microbial ecology of the digestive tract. *J Anim Sci* **87**, 984-993.

Bianchi, A.T.J., Scholten, J.W., vanZijderveld, A.M., vanZijderveld, F.G. and Bokhout, B.A. (1996) Parenteral vaccination of mice and piglets with F4(+) Escherichia coli suppresses the enteric anti-F4 response upon oral infection. *Vaccine* **14**, 199-206.

Biemelt, S., Sonnewald, U., Gaimbacher, P., Willmitzer, L. and Muller, M. (2003) Production of human papillomavirus type 16 virus-like particles in transgenic plants. *J Virol* **77**, 9211-9220.

Bijlsma, I.G. and Bouw, J. (1987) Inheritance of K88-mediated adhesion of *Escherichia coli* to jejunal brush borders in pigs: a genetic analysis. *Vet Res Commun* **11**, 509-518.

Blomberg, L., Henriksson, A. and Conway, P.L. (1993) Inhibition of adhesion of *Escherichia coli* K88 to piglet ileal mucus by *Lactobacillus* spp. *Appl Environ Microbiol* **59**, 34-39.

Bontempo, V., Di Giancamillo, A., Savoini, G., Dell'Orto, V. and Domeneghini, C. (2006) Live yeast dietary supplementation acts upon intestinal morpho-functional aspects and growth in weanling piglets. *Anim Feed Sci Technol* **129**, 224-236.

Boothe, J., Nykiforuk, C., Shen, Y., Zaplachinski, S., Szarka, S., Kuhlman, P., Murray, E., Morck, D. and Moloney, M.M. (2010) Seed-based expression systems for plant molecular farming. *Plant Biotechnol J* **8**, 588-606.

Brandtzaeg, P. (1995) Molecular and cellular aspects of the secretory immunoglobulin system. *Apmis* **103**, 1-19.

Brandtzaeg, P. and Prydz, H. (1984) Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins. *Nature* **311**, 71-74.

Brown, W.R., Kacskovics, I., Amendt, B.A., Blackmore, N.B., Rothschild, M., Shinde, R. and Butler, J.E. (1995) The hinge deletion allelic variant of porcine IgA results from a mutation at the splice acceptor site in the first C alpha intron. *J Immunol* **154**, 3836-3842.

Bruggemann, M., Caskey, H.M., Teale, C., Waldmann, H., Williams, G.T., Surani, M.A. and Neuberger, M.S. (1989) A repertoire of monoclonal antibodies with human heavy-chains from transgenic mice. *Proc Natl Acad Sci USA* **86**, 6709-6713.

Butler, J.E., Wertz, N., Deschacht, N. and Kacskovics, I. (2009) Porcine IgG: structure, genetics, and evolution. *Immunogenetics* **61**, 209-230.

Calinescu, C., Mulhbacher, J., Nadeau, E., Fairbrother, J.M. and Mateescu, M.A. (2005) Carboxymethyl high amylose starch (CM-HAS) as excipient for *Escherichia coli* oral formulations. *Eur J Pharm Biopharm* **60**, 53-60.

Carlson, D., Poulsen, H.D. and Vestergaard, M. (2004) Additional dietary zinc for weaning piglets is associated with elevated concentrations of serum IGF-I. *J Anim Physiol Anim Nutr (Berl)* **88**, 332-339.

Carter, P.J. (2006) Potent antibody therapeutics by design. *Nat Rev Immunol* **6**, 343-357.

Casadevall, A., Dadachova, E. and Pirofski, L. (2004) Passive antibody therapy for infectious diseases. *Nat Rev Microbiol* **2**, 695-703.

Chen, M., Liu, X.W., Wang, Z.K., Song, J., Qi, Q.S. and Wang, P.G. (2005) Modification of plant N-glycans processing: The future of producing therapeutic protein by transgenic plants. *Med Res Rev* **25**, 343-360.

Chen, X., Gao, S., Jiao, X. and Liu, X.F. (2004) Prevalence of serogroups and virulence factors of *Escherichia coli* strains isolated from pigs with postweaning diarrhoea in eastern China. *Vet Microbiol* **103**, 13-20.

Chen, Z.L., Schuler, M.A. and Beachy, R.N. (1986) Functional analysis of regulatory elements in a plant embryo-specific gene. *Proc Natl Acad Sci USA* **83**, 8560-8564.

Cheng, M., Lowe, B., Spencer, T., Ye, X. and Armstrong, C. (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. *In Vitro Cell Dev Biol Plant* **40**, 31-45.

Chernysheva, L.V., Friendship, R.M., Dewey, C.E. and Gyles, C.L. (2004) The effect of dietary chicken egg-yolk antibodies on the clinical response in weaned pigs challenged with a K88(+) *Escherichia coli* isolate. *J Swine Health Prod* **12**, 119-122.

Chintalacharuvu, K.R. and Morrison, S.L. (1997) Production of secretory immunoglobulin A by a single mammalian cell. *Proc Natl Acad Sci USA* **94**, 6364-6368.

Chowdhury, P.S. and Wu, H. (2005) Tailor-made antibody therapeutics. *Methods* **36**, 11-24.

Christien, M. (2010) How pig parts make the world turn. Oxford, England: TED global.

Chrzastek, K. and Piasecki, T. (2011) Reptilian isotypes of antibodies from the aspect of the evolution of the immunoglobulin gene. *Med Weter* **67**, 101-104.

Chung, S.M., Vaidya, M. and Tzfira, T. (2006) Agrobacterium is not alone: gene transfer to plants by viruses and other bacteria. *Trends Plant Sci* **11**, 1-4.

Clerc, S., Hirsch, C., Oggier, D.M., Deprez, P., Jakob, C., Sommer, T. and Aebi, M. (2009) Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum. *J Cell Biol* **184**, 159-172.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* **16**, 735-743.

Coddens, A., Diswall, M., Ångström, J., Breimer, M.E., Goddeeris, B., Cox, E. and Teneberg, S. (2009) Recognition of Blood Group ABH Type 1 Determinants by the FedF Adhesin of F18-fimbriated *Escherichia coli*. *J Biol Chem* **284**, 9713-9726.

Coddens, A., Valis, E., Benktander, J., Ångström, J., Breimer, M.E., Cox, E. and Teneberg, S. (2011) Erythrocyte and porcine intestinal glycosphingolipids recognized by F4 fimbriae of enterotoxigenic *Escherichia coli*. *PLoS One* **6**.

Colson, C., Glover, S.W., Symonds, N. and Stacey, K.A. (1965) The location of the genes for host-controlled modification and restriction in Escherichia coli K-12. *Genetics* **52**, 1043-1050.

Conway, P.L., Welin, A. and Cohen, P.S. (1990) Presence of K88-specific receptors in porcine ileal mucus is age dependent. *Infect Immun* **58**, 3178-3182.

Corbeil, S., Seguin, C. and Trudel, M. (1996) Involvement of the complement system in the protection of mice from challenge with respiratory syncytial virus Long strain following passive immunization with monoclonal antibody 18A2B2. *Vaccine* **14**, 521-525.

Corthésy, B. (2002) Recombinant immunoglobulin A: powerful tools for fundamental and applied research. *Trends Biotechnol* **20**, 65-71.

Corthésy, B. (2003) Recombinant secretory immunoglobulin A in passive immunotherapy: Linking Immunology and biotechnology. *Curr Pharm Biotechnol* **4**, 51-67.

Corthésy, B. and Spertini, F. (1999) Secretory immunoglobulin A: from mucosal protection to vaccine development. *Biol Chem* **380**, 1251-1262.

Costales, A., Son, N.T., Lapar, L., Tiongco, M. and Delgado, C. (2005) Smallholder contract farming of swine in Northern Viet Nam: Study design. In: *Pro-poor livestock policy initiative, a living from livestock*. Rome, Italy: Food and Agricultural Organisation of the United Nations.

Cox, E. and Houvenaghel, A. (1993) Comparison of the *in vitro* adhesion of K88, K99, F41 and P987 positive *Escherichia coli* to intestinal villi of 4- to 5-week-old pigs. *Vet Microbiol* **34**, 7-18.

Cox, E., Schrauwen, E., Cools, V. and Houvenaghel, A. (1991) Experimental induction of diarrhea in newly-weaned piglets. *J Vet Med Ser A-Zentbl Vet Med Reihe A-Physiol Pathol Clin Med* **38**, 418-426.

Cox, K.M., Sterling, J.D., Regan, J.T., Gasdaska, J.R., Frantz, K.K., Peele, C.G., Black, A., Passmore, D., Moldovan-Loomis, C., Srinivasan, M., Cuison, S., Cardarelli, P.M. and Dickey, L.F. (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant Lemna minor. *Nat Biotechnol* **24**, 1591-1597.

Crottet, P., Cottet, S. and Corthésy, B. (1999) Expression, purification and biochemical characterization of recombinant murine secretary component: a novel tool in mucosal immunology. *Biochem J* **341**, 299-306.

Czerkinsky, C. and Holmgren, J. (2010) Topical immunization strategies. *Mucosal Immunol* **3**, 545-555.

Dallas, S.D. and Rolfe, R.D. (1998) Binding of Clostridium difficile toxin A to human milk secretory component. *J Med Microbiol* **47**, 879-888.

De Buck, S., Virdi, V., De Meyer, T., De Wilde, K., Piron, R., Nolf, J., Van Lerberge, E., De Paepe, E. and Depicker, A. (eds) (2011) *Production of camel-like antibodies in plants*. New York: Springer.

De Jaeger, G., Scheffer, S., Jacobs, A., Zambre, M., Zobell, O., Goossens, A., Depicker, A. and Angenon, G. (2002) Boosting heterologous protein production in transgenic dicotyledonous seeds using *Phaseolus vulgaris* regulatory sequences. *Nat Biotech* **20**, 1265-1268.

De Magistnis, M.T. (2006) Mucosal delivery of vaccine antigens and its advantages in pediatrics. *Adv Drug Del Rev* **58**, 52-67.

de Marco, A. (2011) Biotechnological applications of recombinant single-domain antibody fragments. *Microb Cell Fact* **10**, 44.

De Muynck, B., Navarre, C. and Boutry, M. (2010) Production of antibodies in plants: status after twenty years. *Plant Biotechnol J* **8**, 529-563.

De Neve, M., De Buck, S., Jacobs, A., Van Montagu, M. and Depicker, A. (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. *Plant J* **11**, 15-29.

De Wilde, K. (2012) Recombinant protein production in Arabidopsis seeds: Transgene Expression Variation and Transcriptome analysis. In: *Department of Plant Biotechnology and Bioinformatics*. Gent, Belgium: Gent University.

Decker, E.L. and Reski, R. (2008) Current achievements in the production of complex biopharmaceuticals with moss bioreactors. *Bioprocess Biosystems Eng* **31**, 3-9.

Drakakaki, G., Marcel, S., Arcalis, E., Altmann, F., Gonzalez-Melendi, P., Fischer, R., Christou, P. and Stoger, E. (2006) The intracellular fate of a recombinant protein is tissue dependent. *Plant Physiol* **141**, 578-586.

Durandy, A., Kaveri, S.V., Kuijpers, T.W., Basta, M., Miescher, S., Ravetch, J.V. and Rieben, R. (2009) Intravenous immunoglobulins - understanding properties and mechanisms. *Clin Exp Immunol* **158**, 2-13.

Eibl, M.M. (2008) History of Immunoglobulin Replacement. *Immunol Allergy Clin North Am* **28**, 737-764.

Eiffert, H., Quentin, E., Decker, J., Hillemeir, S., Hufschmidt, M., Klingmuller, D., Weber, M.H. and Hilschmann, N. (1984) The primary structure of the human free secretory component and the arrangement of the disulfide bonds. *Hoppe-Seylers Zeitschrift Fur Physiologische Chemie* **365**, 1489-1495.

Fairbrother, J.M., Nadeau, É. and Gyles, C.L. (2005) *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. . *Anim Health Res Rev* **6** 17-39

Faye, L. and Gomord, V. (2010) Success stories in molecular farming-a brief overview. *Plant Biotechnol J* **8**, 525-528.

Field, M., Rao, M.C. and Chang, E.B. (1989) Intestinal electrolyte transport and diarrheal disease (2). *N Engl J Med* **321**, 879-883.

Fischer, R., Drossard, J., Emans, N., Commandeur, U. and Hellwig, S. (1999) Towards molecular farming in the future: pichia pastoris-based production of single-chain antibody fragments. *Biotechnol Appl Biochem* **30 (Pt 2)**, 117-120.

Floss, D.M., Falkenburg, D. and Conrad, U. (2007) Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: an overview. *Transgenic Res* **16**, 315-332.

Fox, J.L. (2003) Puzzling industry response to ProdiGene fiasco. *Nat Biotechnol* **21**, 3-4.

Francis, D.H., Erickson, A.K. and Grange, P.A. (1999) K88 adhesins of enterotoxigenic *Escherichia coli* and their porcine enterocyte receptors. *Adv Exp Med Biol* **473**, 147-154.

Franklin, S.E. and Mayfield, S.P. (2005) Recent developments in the production of human therapeutic proteins in eukaryotic algae. *Expert Opin Biol Ther* **5**, 225-235.

Frenken, L.G.J., van der Linden, R.H.J., Hermans, P.W.J.J., Bos, J.W., Ruuls, R.C., de Geus, B. and Verrips, C.T. (2000) Isolation of antigen specific Llama VHH antibody fragments and their high level secretion by Saccharomyces cerevisiae. *J Biotechnol* **78**, 11-21.

Freyre, F.M., Vázquez, J.E., Ayala, M., Canaán-Haden, L., Bell, H., Rodríguez, I., González, A., Cintado, A. and Gavilondo, J.V. (2000) Very high expression of an anti-carcinoembryonic antigen single chain Fv antibody fragment in the yeast Pichia pastoris. *J Biotechnol* **76**, 157-163.

Garty, B.Z., Ludomirsky, A., Danon, Y.L., Peter, J.B. and Douglas, S.D. (1994) Placental-transfer of immunoglobulin-G subclasses. *Clin Diagn Lab Immunol* **1**, 667-669.

Geenen, P.L., Van der Meulen, J., Bouma, A. and De Jong, M.C.M. (2004) Estimating transmission parameters of F4+ *E. coli* for F4-receptor-positive and -negative piglets: one-to-one transmission experiment. *Epidemiol Infect* **132**, 1039-1048.

Geenen, P.L., Van der Meulen, J., Bouma, A., Engel, B., Heesterbeek, J.A.P. and De Jong, M.C.M. (2007) Classification of temporal profiles of F4+*E. coli* shedding and faecal dry matter in experimental post-weaning diarrhoea of pigs. *Epidemiol Infect* **135**, 1001-1009.

Geyer, B.C., Kannan, L., Cherni, I., Woods, R.R., Soreq, H. and Mor, T.S. (2010) Transgenic plants as a source for the bioscavenging enzyme, human butyrylcholinesterase. *Plant Biotechnol J* **8**, 873-886.

Githigia, S.M., Okuthe, S. and Diop, B. (2012) Pig Sector Kenya. In: *FAO Animal Production and Health Livestock Country Reviews No. 3*. Rome, Italy: Food and Agriculture Organisation of the United Nations, Rome.

Giugliano, L.G., Ribeiro, S.T.G., Vainstein, M.H. and Ulhoa, C.J. (1995) Free secretory component and lactoferrin of human milk inhibit the adhesion of enterotoxigenic *Escherichia coli*. *J Med Microbiol* **42**, 3-9.

Gleba, Y., Klimyuk, V. and Marillonnet, S. (2005) Magnifection--a new platform for expressing recombinant vaccines in plants. *Vaccine* **23**, 2042-2048.

Goldsby, R., Kindt, T., Osborne, B. and Kuby, J. (2003) *Immunology*. New York:W.H. Freeman and Company, New York.

Gonik, B. (2011) Passive immunization: the forgotten arm of immunologically based strategies for disease containment. *Am J Obstet Gynecol* **205**, 444.e441-444.e446.

Gonzalez, E.A., Vazquez, F., Garabal, J.I. and Blanco, J. (1995) Isolation of K88 antigen variants (ab, ac, ad) from porcine enterotoxigenic *Escherichia coli* belonging to different serotypes. *Microbiol Immunol* **39**, 937-942.

Grange, P.A., Mouricout, M.A., Levery, S.B., Francis, D.H. and Erickson, A.K. (2002) Evaluation of receptor binding specificity of *Escherichia coli* K88 (F4) fimbrial adhesin variants using porcine serum transferrin and glycosphingolipids as model receptors. *Infect Immun* **70**, 2336-2343.

Greenberg, A.S., Avila, D., Hughes, M., Hughes, A., McKinney, E.C. and Flajnik, M.F. (1995) A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. *Nature* **374**, 168-173.

Gupta, R., Jung, E. and Brunak, S. (2004) Prediction of N-glycosylation sites in human proteins.

Hahn, E., Wild, P., Schraner, E.M., Bertschinger, H.U., Haner, M., Muller, S.A. and Aebi, U. (2000) Structural analysis of F18 fimbriae expressed by porcine toxigenic *Escherichia coli*. *J Struct Biol* **132**, 241-250.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman, N. and Hamers, R. (1993) Naturally occurring antibodies devoid of light chains. *Nature* **363**, 446-448.

Hara-Nishimura, I., Shimada, T., Hatano, K., Takeuchi, Y. and Nishimura, M. (1998) Transport of storage proteins to protein storage vacuoles is mediated by large precursor-accumulating vesicles. *Plant Cell* **10**, 825-836.

Harkensee, C., Brodlie, M., Embleton, N.D. and McKean, M. (2006) Passive immunisation of preterm infants with palivizumab against RSV infection. *J Infect* **52**, 2-8.

Harmsen, M.M. and De Haard, H.J. (2007) Properties, production, and applications of camelid single-domain antibody fragments. *Appl Microbiol Biotechnol* **77**, 13-22.

Harmsen, M.M., van Solt, C.B., Hoogendoorn, A., van Zijderveld, F.G., Niewold, T.A. and van der Meulen, J. (2005) *Escherichia coli* F4 fimbriae specific llama single-domain antibody fragments effectively inhibit bacterial adhesion *in vitro* but poorly protect against diarrhoea. *Vet Microbiol* **111**, 89-98.

Hassanzadeh-Ghassabeh, G., Saerens, D. and Muyldermans, S. (2011) Generation of Anti-infectome/Anti-proteome Nanobodies. *Methods Mol Biol* **790**, 239-259.

Hasselquist, D. and Nilsson, J.A. (2009) Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. *Philos Trans R Soc Lond B Biol Sci* **364**, 51-60.

Hellwig, S., Drossard, J., Twyman, R.M. and Fischer, R. (2004) Plant cell cultures for the production of recombinant proteins. *Nat Biotechnol* **22**, 1415-1422.

Heo, J.M., Opapeju, F.O., Pluske, J.R., Kim, J.C., Hampson, D.J. and Nyachoti, C.M. (2012) Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *J Anim Physiol Anim Nutr*, (available online, ahead of print).

Hiatt, A., Cafferkey, R. and Bowdish, K. (1989) Production of antibodies in transgenic plants. *Nature* **342**, 76-78.

Hirsch, C., Gauss, R., Horn, S.C., Neuber, O. and Sommer, T. (2009) The ubiquitylation machinery of the endoplasmic reticulum. *Nature* **458**, 453-460.

Hmila, I., Abdallah R, B.A.-B., Saerens, D., Benlasfar, Z., Conrath, K., Ayeb, M.E., Muyldermans, S. and Bouhaouala-Zahar, B. (2008) VHH, bivalent domains and chimeric Heavy chain-only antibodies with high neutralizing efficacy for scorpion toxin AahI'. *Mol Immunol* **45**, 3847-3856.

Hohl, I., Robinson, D.G., Chrispeels, M.J. and Hinz, G. (1996) Transport of storage proteins to the vacuole is mediated by vesicles without a clathrin coat. *J Cell Sci* **109 (Pt 10)**, 2539-2550.

Højberg, O., Canibe, N., Poulsen, H.D., Hedemann, M.S. and Jensen, B.B. (2005) Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. *Appl Environ Microbiol* **71**, 2267-2277.

Holliger, P. and Hudson, P.J. (2005) Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* **23**, 1126-1136.

Holmgren, J. and Czerkinsky, C. (2005) Mucosal immunity and vaccines. *Nat Med* **11**, S45-S53.

Hong, T.T.T., Linh, N.Q., Ogle, B. and Lindberg, J.E. (2006) Survey on the prevalence of diarrhoea in pre-weaning piglets and on feeding systems as contributing risk factors in smallholdings in Central Vietnam. *Trop Anim Health Prod* **38**, 397-405.

Hood, E.E., Woodard, S.L. and Horn, M.E. (2002) Monoclonal antibody manufacturing in transgenic plants — myths and realities. *Curr Opin Biotechnol* **13**, 630-635.

Horwitz, A.H., Chang, C.P., Better, M., Hellstrom, K.E. and Robinson, R.R. (1988) Secretion of functional antibody and Fab fragment from yeast cells. *Proc Natl Acad Sci U S A* **85**, 8678-8682.

Houdebine, L.-M. (2009) Production of pharmaceutical proteins by transgenic animals. *Comp Immunol, Microbiol Infect Dis* **32**, 107-121.

Houdebine, L.M. (2002) Antibody manufacture in transgenic animals and comparisons with other systems. *Curr Opin Biotechnol* **13**, 625-629.

Hsu, J.L. and Safdar, N. (2011) Polyclonal immunoglobulins and hyperimmune globulins in prevention and management of infectious diseases. *Infect Dis Clin North Am* **25**, 773-788.

Huang, Z., Phoolcharoen, W., Lai, H., Piensook, K., Cardineau, G., Zeitlin, L., Whaley, K.J., Arntzen, C.J., Mason, H.S. and Chen, Q. (2010) High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. *Biotechnol Bioeng* **106**, 9-17.

Hughes, G.J., Frutiger, S., Savoy, L.A., Reason, A.J., Morris, H.R. and Jaton, J.C. (1997) Human free secretory component is composed of the first 585 amino acid residues of the polymeric immunoglobulin receptor. *FEBS Lett* **410**, 443-446.

Hurley, W.L. and Theil, P.K. (2011) Perspectives on immunoglobulins in colostrum and milk. *Nutrients* **3**, 442-474.

Huyghebaert, N., Snoeck, V., Vermeire, A., Cox, E., Goddeeris, B.M. and Remon, J.P. (2005) Development of an enteric-coated pellet formulation of F4 fimbriae for oral vaccination of suckling piglets against enterotoxigenic *Escherichia coli* infections. *Eur J Pharm Biopharm* **59**, 273-281.

Imberechts, H., Wild, P., Charlier, G., De Greve, H., Lintermans, P. and Pohl, P. (1996) Characterization of F18 fimbrial genes FedE and FedF involved in adhesion and length of enterotoxemic *Escherichia coli* strain 107/86. *Microb Pathog* **21**, 183-192.

Jin, L.Z., Marquardt, R.R. and Zhao, X. (2000) A strain of *Enterococcus faecium* (18C23) inhibits adhesion of enterotoxigenic *Escherichia coli* K88 to porcine small intestine mucus. *Appl Environ Microbiol* **66**, 4200-4204.

Joensuu, J.J., Kotiaho, M., Riipi, T., Snoeck, V., Palva, E.T., Teeri, T.H., Lang, H., Cox, E., Goddeeris, B.M. and Niklander-Teeri, V. (2004) Fimbrial subunit protein FaeG expressed in transgenic tobacco inhibits the binding of F4ac enterotoxigenic *Escherichia coli* to porcine enterocytes. *Transgenic Res* **13**, 295-298.

Joensuu, J.J., Kotiaho, M., Teeri, T.H., Valmu, L., Nuutila, A.M., Oksman-Caldentey, K.M. and Niklander-Teeri, V. (2006a) Glycosylated F4 (K88) fimbrial adhesin FaeG expressed in barley endosperm induces ETEC-neutralizing antibodies in mice. *Transgenic Res* **15**, 359-373.

Joensuu, J.J., Verdonck, F., Ehrström, A., Peltola, M., Siljander-Rasi, H., Nuutila, A.M., Oksman-Caldentey, K.M., Teeri, T.H., Cox, E., Goddeeris, B.M. and Niklander-Teeri, V. (2006b) F4 (K88) fimbrial adhesin FaeG expressed in alfalfa reduces F4+ enterotoxigenic *Escherichia coli* excretion in weaned piglets. *Vaccine* **24**, 2387-2394.

Johansen, F.E., Braathen, R. and Brandtzaeg, P. (2000) Role of J chain in secretory immunoglobulin formation. *Scand J Immunol* **52**, 240-248.

Johansen, F.E., Norderhaug, I.N., Roe, M., Sandlie, I. and Brandtzaeg, P. (1999) Recombinant expression of polymeric IgA: incorporation of J chain and secretory component of human origin. *Eur J Immunol* **29**, 1701-1708.

Kaetzel, C.S. (2005) The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces. *Immunol Rev* **206**, 83-99.

Kapila, J., DeRycke, R., VanMontagu, M. and Angenon, G. (1997) An Agrobacterium-mediated transient gene expression system for intact leaves. *Plant Sci* **122**, 101-108.

Kapitany, R.A. and Zebrowski, E.J. (1973) A high resolution PAS stain for polyacrylamide gel electrophoresis. *Anal Biochem* **56**, 361-369.

Karimi, M., De Meyer, B. and Hilson, P. (2005) Modular cloning in plant cells. *Trends Plant Sci* **10**, 103-105.

Karnoup, A.S., Turkelson, V. and Anderson, W.H.K. (2005) O-Linked glycosylation in maize-expressed human IgA1. *Glycobiology* **15**, 965-981.

Katouli, M., Melin, L., Jensen-Waern, M., Wallgren, P. and Mollby, R. (1999) The effect of zinc oxide supplementation on the stability of the intestinal flora with special reference to composition of coliforms in weaned pigs. *J Appl Microbiol* **87**, 564 - 573.

Kenworthy, R. (1976) Observations on the effects of weaning in the young pig. Clinical and histopathological studies of intestinal function and morphology. *Res Vet Sci* **21**, 69-75.

Khan, I., Twyman, R.M., Arcalis, E. and Stoger, E. (2012) Using storage organelles for the accumulation and encapsulation of recombinant proteins. *Biotechnol J*, (available online, ahead of print).

Kiarie, E.G., Slominski, B.A., Krause, D.O. and Nyachoti, C.M. (2008) Nonstarch polysaccharide hydrolysis products of soybean and canola meal protect against enterotoxigenic *Escherichia coli* in piglets. *J Nutr* **138**, 502-508.

Kiarie, E.G., Slominski, B.A. and Nyachoti, C.M. (2010) Effect of products derived from hydrolysis of wheat and flaxseed non starch polysaccharides by carbohydrase enzymes on net absorption in enterotoxigenic *Escherichia coli* (K88) challenged piglet jejunal segments. *Anim Sci J* **81**, 63-71.

Kim, J.C., Hansen, C.F., Mullan, B.P. and Pluske, J.R. (2012) Nutrition and pathology of weaner pigs: Nutritional strategies to support barrier function in the gastrointestinal tract. *Anim Feed Sci Technol* **173**, 3-16.

Ko, K. and Koprowski, H. (2005) Plant biopharming of monoclonal antibodies. *Virus Res* **111**, 93-100.

Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495-497.

Koncz, C. and Schell, J. (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol Gen Genet* **204**, 383-396.

Kosten, T. and Owens, S.M. (2005) Immunotherapy for the treatment of drug abuse. *Pharmacol Ther* **108**, 76-85.

Kumura, B.H., Sone, T., Shimazaki, K. and Kobayashi, E. (2000) Sequence analysis of porcine polymeric immunoglobulin receptor from mammary epithelial cells present in colostrum. *J Dairy Res* **67**, 631-636.

Kunhareang, S., Zhou, H. and Hickford, J.G.H. (2009) Novel sequence of the porcine IGHA gene. *Mol Immunol* **47**, 147-148.

Lau, O.S. and Sun, S.S.M. (2009) Plant seeds as bioreactors for recombinant protein production. *Biotechnol Adv* **27**, 1015-1022.

Lauwereys, M., Arbabi Ghahroudi, M., Desmyter, A., Kinne, J., Holzer, W., De Genst, E., Wyns, L. and Muyldermans, S. (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO J* **17**, 3512-3520.

Lecce, J.G. (1983) Dietary regimen, rotavirus, and hemolytic enteropathogenic *Escherichia col*i in weanling diarrhea of pigs. *Ann Rech Vet* **14**, 463 - 468.

Lewis, M.J., Wagner, B., Irvine, R.M. and Woof, J.M. (2010) IgA in the horse: cloning of equine polymeric Ig receptor and J chain and characterization of recombinant forms of equine IgA. *Mucosal Immunol* **3**, 610-621.

Li, J. and Zhu, Z. (2010) Research and development of next generation of antibody-based therapeutics. *Acta Pharmacologica Sinica* **31**, 1198-1207.

Loos, A., Van Droogenbroeck, B., Hillmer, S., Grass, J., Kunert, R., Cao, J., Robinson, D.G., Depicker, A. and Steinkellner, H. (2011a) Production of monoclonal antibodies with a controlled N-glycosylation pattern in seeds of *Arabidopsis thaliana*. *Plant Biotechnol J* **9**, 179-192.

Loos, A., Van Droogenbroeck, B., Hillmer, S., Grass, J., Pabst, M., Castilho, A., Kunert, R., Liang, M., Arcalis, E., Robinson, D.G., Depicker, A. and Steinkellner, H. (2011b) Expression of antibody fragments with a controlled N-glycosylation pattern and induction of ER-derived vesicles in seeds of *Arabidopsis thaliana*. *Plant Physiol* **155**, 2036-2048.

Lullau, E., Heyse, S., Vogel, H., Marison, I., vonStockar, U., Kraehenbuhl, J.P. and Corthesy, B. (1996) Antigen binding properties of purified immunoglobulin A and reconstituted secretory immunoglobulin A antibodies. *J Biol Chem* **271**, 16300-16309.

Ma, J.K., Hiatt, A., Hein, M., Vine, N.D., Wang, F., Stabila, P., van Dolleweerd, C., Mostov, K. and Lehner, T. (1995) Generation and assembly of secretory antibodies in plants. *Science* **268**, 716-719.

Ma, J.K.C., Hunjan, M., Smith, R., Kelly, C. and Lehner, T. (1990) An investigation into the mechanism of protection by local passive immunization with monoclonal antibodies against *Streptococcus mutans*. *Infect Immun* **58**, 3407-3414.

Macpherson, A.J., Geuking, M.B. and McCoy, K.D. (2012) Homeland Security: IgA immunity at the frontiers of the body. *Trends Immunol* **33**, 160-167.

Madec, F., Bridoux, N., Bounaix, S., Cariolet, R., Duval-Iflah, Y., Hampson, D.J. and Jestin, A. (2000) Experimental models of porcine post-weaning colibacillosis and their relationship to post-weaning diarrhoea and digestive disorders as encountered in the field. *Vet Microbiol* **72**, 295-310.

Marquardt, R.R., Jin, L.Z., Kim, J.-W., Fang, L., Frohlich, A.A. and Baidoo, S.K. (1999) Passive protective effect of egg-yolk antibodies against enterotoxigenic *Escherichia coli* K88+ infection in neonatal and early-weaned piglets. *FEMS Immunol Med Microbiol* **23**, 283-288.

Mason, H.S., Palmer, K.E., Hefferon, K.L., Mor, T.S. and Arntzen, C. (2002) Gemini virus vectors for gene expression in plants. . (Patent, U.S. ed). United State of America Boyce Thompson Institte for plant research, Ithaca, Ny (US).

Mayfield, S.P. and Franklin, S.E. (2005) Expression of human antibodies in eukaryotic micro-algae. *Vaccine* **23**, 1828-1832.

McCormick, A.A., Reddy, S., Reinl, S.J., Cameron, T.I., Czerwinkski, D.K., Vojdani, F., Hanley, K.M., Garger, S.J., White, E.L., Novak, J., Barrett, J., Holtz, R.B., Tuse, D. and Levy, R. (2008) Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a phase I clinical study. *Proc Natl Acad Sci U S A* **105**, 10131-10136.

Melin, L., Katouli, M., Lindberg, A., Fossum, C. and Wallgren, P. (2000) Weaning of piglets. Effects of an exposure to a pathogenic strain of *Escherichia coli*. *J Vet Med B Infect Dis Vet Public Health* **47**, 663-675.

Melin, L. and Wallgren, P. (2002) Aspects on feed related prophylactic measures aiming to prevent post weaning diarrhoea in pigs. *Acta vet scand* **43**, 231-245.

Melkebeek, V., Rasschaert, K., Bellot, P., Tilleman, K., Favoreel, H., Deforce, D., De Geest, B.G., Goddeeris, B.M. and Cox, E. (2012) Targeting aminopeptidase N, a newly identified receptor for F4ac fimbriae, enhances the intestinal mucosal immune response. *Mucosal Immunol*, Epub ahead of print.

Mikschofsky, H. and Broer, I. (2011) Feasibility of Pisum sativum as an expression system for pharmaceuticals. *Transgenic Res*, 1-10.

Mol, O. and Oudega, B. (1996) Molecular and structural aspects of fimbriae biosynthesis and assembly in *Escherichia coli*. *FEMS Microbiol Rev* **19**, 25-52.

Mol, O., Oudhuis, W.C., Fokkema, H. and Oudega, B. (1996) The N-terminal beta-barrel domain of the *Escherichia coli* K88 periplasmic chaperone FaeE determines fimbrial subunit recognition and dimerization. *Mol Microbiol* **22**, 379-388.

Mol, O., Oudhuis, W.C., Oud, R.P.C., Sijbrandi, R., Luirink, J., Harms, N. and Oudega, B. (2001) Biosynthesis of K88 fimbriae in *Escherichia coli*: Interaction of tip-subunit FaeC with the periplasmic chaperone FaeE and the outer membrane usher FaeD. *J Mol Microbiol Biotechnol* **3**, 135-142.

Moon, H.W. and Bunn, T.O. (1993) Vaccines for preventing enterotoxigenic *Escherichia coli* infections in farm animals. *Vaccine* **11**, 213-220.

Morandini, F., Avesani, L., Bortesi, L., Van Droogenbroeck, B., De Wilde, K., Arcalis, E., Bazzoni, F., Santi, L., Brozzetti, A., Falorni, A., Stoger, E., Depicker, A. and Pezzotti, M. (2011) Non-food/feed seeds as biofactories for the high-yield production of recombinant pharmaceuticals. *Plant Biotechnol J* **9**, 911-921.

Morris, D.H. (2007) *Flax- A Health and Nutrition Primer*. Canada:Flax council of Canada.

Muntz, K. (1998) Deposition of storage proteins. *Plant Mol Biol* **38**, 77-99.

Muyldermans, S. (2001) Single domain camel antibodies: current status. *Rev Mol Biotechnol* **74**, 277-302.

Muyldermans, S., Cambillau, C. and Wyns, L. (2001) Recognition of antigens by single domain antibody fragments: the superfluous luxury of paired domains. *Trends Biochem Sci* **26**, 230-235.

Mynott, T.L., Luke, R.K.J. and Chandler, D.S. (1996) Oral administration of protease inhibits enterotoxigenic Escherichia coli receptor activity in piglet small intestine. *Gut* **38**, 28-32.

Nagels, B., Santens, F., Weterings, K., Van Damme, E.J.M. and Callewaert, N. (2011) Improved sample preparation for CE-LIF analysis of plant N-glycans. *Electrophoresis* **32**, 3482-3490.

Nagy, B. and Fekete, P.Z. (1999) Enterotoxigenic *Escherichia coli (*ETEC) in farm animals. *Vet Res* **30**, 259-284.

Naz, R.K. and Rajesh, C. (2004) Passive immunization for immunocontraception: Lessons learned from infectious diseases. *Front Biosci* **9**, 2457-2465.

Niewold, T.A., van Dijk, A.J., Geenen, P.L., Roodink, H., Margry, R. and van der Meulen, J. (2007) Dietary specific antibodies in spray-dried immune plasma prevent enterotoxigenic *Escherichia coli* F4 (ETEC) post weaning diarrhoea in piglets. *Vet Microbiol* **124**, 362-369.

Nuttall, J., Vine, N., Hadlington, J.L., Drake, P., Frigerio, L. and Ma, J.K.C. (2002) ER-resident chaperone interactions with recombinant antibodies in transgenic plants. *Eur J Biochem* **269**, 6042-6051.

O'Doherty, J.V., Nolan, C.S., Callan, J.J. and McCarthy, P. (2004) The interaction between lactofeed level and soya-bean meal on growth performance of weanling pigs. *Animal Science* **78**, 419-427.

O'Doherty, J.V., Nolan, C.S. and McCarthy, P. (2005) Interaction between lactose levels and antimicrobial growth promoters on growth performance of weanling pigs. *J Sci Food Agric* **85**, 371-380.

Obembe, O.O., Popoola, J.O., Leelavathi, S. and Reddy, S.V. (2011) Advances in plant molecular farming. *Biotechnol Adv* **29**, 210-222.

Obregon, P., Chargelegue, D., Drake, P.M.W., Prada, A., Nuttall, J., Frigerio, L. and Ma, J.K.C. (2006) HIV-1 p24–immunoglobulin fusion molecule: a new strategy for plant-based protein production. *Plant Biotechnol J* **4**, 195-207.

Oleksiewicz, M.B., Nagy, G. and Nagy, E. (2012) Anti-bacterial monoclonal antibodies: Back to the future? *Arch Biochem Biophys*, ahead of print.

Osek, J. (1999) Prevalence of virulence factors of *Escherichia coli* strains isolated from diarrheic and healthy piglets after weaning. *Vet Microbiol* **68**, 209-217.

Owusu-Asiedu, A., Baidoo, S.K., Nyachoti, C.M. and Marquardt, R.R. (2002a) Response of early-weaned pigs to spray-dried porcine or animal plasma-based diets supplemented with egg-yolk antibodies against enterotoxigenic *Escherichia coli*. *J Anim Sci* **80**, 2895-2903.

Owusu-Asiedu, A., Baidoot, S.K., Nyachoti, C.M. and Marquardt, R.R. (2002b) Response of early-weaned pigs to spray-dried porcine or animal plasma-based diets supplemented with egg-yolk antibodies against enterotoxigenic Escherichia colil. *J Anim Sci* **80**, 2895-2903.

Paul, M. and Ma, J.K.C. (2011) Plant-made pharmaceuticals: Leading products and production platforms. *Biotechnol Appl Biochem* **58**, 58-67.

Paul, M., van Dolleweerd, C., Drake, P.M., Reljic, R., Thangaraj, H., Barbi, T., Stylianou, E., Pepponi, I., Both, L., Hehle, V., Madeira, L., Inchakalody, V., Ho, S., Guerra, T. and Ma, J.K. (2011) Molecular Pharming: future targets and aspirations. *Hum Vaccin* **7**, 375-382.

Paz, M.M., Martinez, J.C., Kalvig, A.B., Fonger, T.M. and Wang, K. (2006) Improved cotyledonary node method using an alternative explant derived from mature seed

for efficient Agrobacterium-mediated soybean transformation. *Plant Cell Rep* **25**, 206-213.

Perrin, Y., Vaquero, C., Gerrard, I., Sack, M., Drossard, J., Stoger, E., Christou, P. and Fischer, R. (2000) Transgenic pea seeds as bioreactors for the production of a single-chain Fv fragment (scFV) antibody used in cancer diagnosis and therapy. *Mol Breed* **6**, 345-352.

Peters, J. and Stoger, E. (2011) Transgenic crops for the production of recombinant vaccines and anti-microbial antibodies. *Hum Vaccin* **7**, 367-374.

Petruccelli, S., Otegui, M.S., Lareu, F., Dinh, O.T., Fitchette, A.-C., Circosta, A., Rumbo, M., Bardor, M., Carcamo, R., Gomord, V. and Beachy, R.N. (2006) A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and sorted to protein storage vacuoles in seeds. *Plant Biotechnol J* **4**, 511-527.

Philibert, P., Stoessel, A., Wang, W., Sibler, A.P., Bec, N., Larroque, C., Saven, J.G., Courtete, J., Weiss, E. and Martineau, P. (2007) A focused antibody library for selecting scFvs expressed at high levels in the cytoplasm. *BMC Biotechnol* **7**, 81.

Pierce, K.M., Callan, J.J., McCarthy, P. and O'Doherty, J.V. (2004) Effects of high dietary concentration of lactose and increased soya-bean meal inclusion in starter diets for piglets. *Animal Science* **79**, 445-452.

Pluske, J.R., Hampson, D.J. and Williams, I.H. (1997) Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livestock Production Science* **51**, 215-236.

Pogue, G.P., Vojdani, F., Palmer, K.E., Hiatt, E., Hume, S., Phelps, J., Long, L., Bohorova, N., Kim, D., Pauly, M., Velasco, J., Whaley, K., Zeitlin, L., Garger, S.J., White, E., Bai, Y., Haydon, H. and Bratcher, B. (2010) Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. *Plant Biotechnol J* **8**, 638-654.

Polowick, P.L., Quandt, J. and Mahon, J.D. (2000) The ability of pea transformation technology to transfer genes into peas adapted to western Canadian growing conditions. *Plant Sci* **153**, 161-170.

Porter, P., Noakes, D.E. and Allen, W.D. (1970) Secretory IgA and Antibodies to Escherichia coli in Porcine Colostrum and Milk and their Significance in the Alimentary Tract of the Young Pig. *Immunology* **18**, 245 - 257.

Potgieter, T.I., Cukan, M., Drummond, J.E., Houston-Cummings, N.R., Jiang, Y., Li, F., Lynaugh, H., Mallem, M., McKelvey, T.W. and Mitchell, T. (2009) Production of monoclonal antibodies by glycoengineered Pichia pastoris. *J Biotechnol* **139**, 318-325.

Raab, C.P. (2011) Passive immunization. *Prim Care* **38**, 681-691, viii.

Rademacher, T., Sack, M., Arcalis, E., Stadlmann, J., Balzer, S., Altmann, F., Quendler, H., Stiegler, G., Kunert, R., Fischer, R. and Stoger, E. (2008) Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. *Plant Biotechnol J* **6**, 189-201.

Ramessar, K., Rademacher, T., Sack, M., Stadlmann, J., Platis, D., Stiegler, G., Labrou, N., Altmann, F., Ma, J., Stoger, E., Capell, T. and Christou, P. (2008) Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. *Proc Natl Acad Sci USA* **105**, 3727-3732.

Rasschaert, K., Verdonck, F., Goddeeris, B.M., Duchateau, L. and Cox, E. (2007) Screening of pigs resistant to F4 enterotoxigenic *Escherichia coli* (ETEC) infection. *Vet Microbiol* **123**, 249-253.

Reilly, R.M., Domingo, R. and Sandhu, J. (1997) Oral delivery of antibodies - Future pharmacokinetic trends. *Clin Pharmacokinet* **32**, 313-323.

Rietveld, E., Steyerberg, E.W., Polder, J.J., Veeze, H.J., Vergouwe, Y., Huysman, M.W.A., de Groot, R. and Moll, H.A. (2010) Passive immunisation against respiratory syncytial virus: a cost-effectiveness analysis. *Arch Dis Child* **95**, 493-498.

Riising, H.J., Murmans, M. and Witvliet, M. (2005) Protection against neonatal *Escherichia coli* diarrhoea in pigs by vaccination of sows with a new vaccine that contains purified enterotoxic *E. coli* virulence factors F4ac, F4ab, F5 and F6 fimbrial antigens and heat-labile *E. coli* enterotoxin (LT) toxoid. *J Vet Med B* **52**, 296-300.

Rindisbacher, L., Cottet, S., Wittek, R., Kraehenbuhl, J.P. and Corthesy, B. (1995) Production of human secretory component with dimeric IgA binding-capacity using viral expression systems. *J Biol Chem* **270**, 14220-14228.

Rippinger, P., Bertschinger, H.U., Imberechts, H., Nagy, B., Sorg, I., Stamm, M., Wild, P. and Wittig, W. (1995) Designations F18ab and F18ac for the related fimbrial types F107, 2134P and 8813 of *Escherichia coli* isolated from porcine postweaning diarrhoea and from oedema disease. *Vet Microbiol* **45**, 281-295.

Roselli, M., Finamore, A., Britti, M.S., Bosi, P., Oswald, I. and Mengheri, E. (2005) Alternatives to in-feed antibiotics in pigs: Evaluation of probiotics, zinc or organic acids as protective agents for the intestinal mucosa. A comparison of in vitro and in vivo results. *Anim Res* **54**, 203-218.

Rybicki, E.P. (2010) Plant-made vaccines for humans and animals. *Plant Biotechnol J* **8**, 620-637.

Saalbach, I., Riehl, M., Giersberg, M., Kumlehn, J. and Falkenburg, D. (2007) Production of recombinant antibodies in pea seeds and their oral application in piglets. In: *Biotechnology and Sustainable Agriculture 2006 and Beyond* (Xu, Z., Li, J., Xue, Y. and Yang, W. eds), pp. 399-402. Dordrecht: Springer.

Saerens, D., Ghassabeh, G.H. and Muyldermans, S. (2008) Single-domain antibodies as building blocks for novel therapeutics. *Curr Opin Pharm* **8**, 600-608.

Saerens, D., Pellis, M., Loris, R., Pardon, E., Dumoulin, M., Matagne, A., Wyns, L., Muyldermans, S. and Conrath, K. (2005) Identification of a universal VHH framework to graft non-canonical antigen-binding loops of camel single-domain antibodies. *J Mol Biol* **352**, 597-607.

Sainsbury, F. and Lomonossoff, G.P. (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiol* **148**, 1212-1218.

Sainsbury, F., Sack, M., Stadlmann, J., Quendler, H., Fischer, R. and Lomonossoff, G.P. (2010) Rapid transient production in plants by replicating and non-replicating vectors yields high quality functional anti-HIV antibody. *PLoS One* **5**, e13976.

Sainsbury, F., Thuenemann, E.C. and Lomonossoff, G.P. (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol J* **7**, 682-693.

Saint-Jore-Dupas, C., Faye, L. and Gomord, V. (2007) From planta to pharma with glycosylation in the toolbox. *Trends Biotechnol* **25**, 317-323.

Schirrmann, T., Al-Halabi, L., Dubel, S. and Hust, M. (2008) Production systems for recombinant antibodies. *Front Biosci* **13**, 4576-4594.

Schmidt, F.R. (2004) Recombinant expression systems in the pharmaceutical industry. *Appl Microbiol Biotechnol* **65**, 363-372.

Sellwood, R. (1979) Escherichia coli diarrhoea in pigs with or without the K88 receptor. *Vet Rec* **105**, 228-230.

Sethuraman, N. and Stadheim, T.A. (2006) Challenges in therapeutic glycoprotein production. *Curr Opin Biotechnol* **17**, 341-346.

Setia, A., Bhandari, S.K., House, J.D., Nyachoti, C.M. and Krause, D.O. (2009) Development and *in vitro* evaluation of an *Escherichia coli* probiotic able to inhibit the growth of pathogenic *Escherichia coli* K88. *J Anim Sci* **87**, 2005-2012.

Sharp, J.M. and Doran, P.M. (2001) Strategies for enhancing monoclonal antibody accumulation in plant cell and organ cultures. *Biotechnol Prog* **17**, 979-992.

Simmons, L.C., Reilly, D., Klimowski, L., Raju, T.S., Meng, G., Sims, P., Hong, K., Shields, R.L., Damico, L.A., Rancatore, P. and Yansura, D.G. (2002) Expression of full-length immunoglobulins in *Escherichia coli*: rapid and efficient production of aglycosylated antibodies. *J Immunol Methods* **263**, 133-147.

Sletta, H., Nedal, A., Aune, T.E.V., Hellebust, H., Hakvag, S., Aune, R., Ellingsen, T.E., Valla, S. and Brautaset, T. (2004) Broad-host-range plasmid pJB658 can be used for industrial-level production of a secreted host-toxic single-chain antibody fragment in Escherichia coli. *Appl Environ Microbiol* **70**, 7033-7039.

Sletta, H., Tondervik, A., Hakvag, S., Aune, T.E., Nedal, A., Aune, R., Evensen, G., Valla, S., Ellingsen, T.E. and Brautaset, T. (2007) The presence of N-terminal secretion signal sequences leads to strong stimulation of the total expression levels of three tested medically important proteins during high-cell-density cultivations of Escherichia coli. *Appl Environ Microbiol* **73**, 906-912.

Smeds, A., Pertovaara, M., Timonen, T., Pohjanvirta, T., Pelkonen, S. and Palva, A. (2003) Mapping the binding domain of the F18 fimbrial adhesin. *Infect Immun* **71**, 2163-2172.

Smith, G.P. (1985) Filamentous fusion phage - novel expression vectors that display cloned antigens on the virion surface. *Science* **228**, 1315-1317.

Smith, H.W. and Linggood, M.A. (1971) Observations on the pathogenic properties of the K88, Hly and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. *J Med Microbiol* **4**, 467-485.

Snoeck, V., Cox, E., Verdonck, F., Joensuu, J.J. and Goddeeris, B.M. (2004) Influence of porcine intestinal pH and gastric digestion on antigenicity of F4 fimbriae for oral immunisation. *Vet Microbiol* **98**, 45-53.

Snoeck, V., Huyghebaert, N., Cox, E., Vermeire, A., Vancaeneghem, S., Remon, J.P. and Goddeeris, B.M. (2003) Enteric-coated pellets of F4 fimbriae for oral vaccination of suckling piglets against enterotoxigenic *Escherichia coli* infections. *Vet Immunol Immunopathol* **96**, 219-227.

Sola-Oriol, D., Roura, E. and Torrallardona, D. (2011) Feed preference in pigs: Effect of selected protein, fat, and fiber sources at different inclusion rates. *J Anim Sci* **89**, 3219-3227.

Stirling, C.M.A., Charleston, B., Takamatsu, H., Claypool, S., Lencer, W., Blumberg, R.S. and Wileman, T.E. (2005) Characterization of the porcine neonatal Fc receptor - potential use for trans-epithelial protein delivery. *Immunology* **114**, 542-553.

Stoger, E., Vaquero, C., Torres, E., Sack, M., Nicholson, L., Drossard, J., Williams, S., Keen, D., Perrin, Y., Christou, P. and Fischer, R. (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Mol Biol* **42**, 583-590.

Streatfield, S.J. (2006) Mucosal immunization using recombinant plant-based oral vaccines. *Methods* **38**, 150-157.

Streatfield, S.J. (2007) Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnol J* **5**, 2-15.

Strugnell, R.A. and Wijburg, O.L.C. (2010) The role of secretory antibodies in infection immunity. *Nat Rev Microbiol* **8**, 656-667.

Sunilkumar, G., Waghela, S.D., Campbell, L.M. and Rathore, K.S. (2009) Expression of anti-K99 scFv in transgenic rice tissues and its functional characterization. *Transgenic Res* **18**, 347-360.

Takagi, H., Hiroi, T., Hirose, S., Yang, L. and Takaiwa, F. (2010) Rice seed ER-derived protein body as an efficient delivery vehicle for oral tolerogenic peptides. *Peptides* **31**, 1421-1425.

Taube, V.A., Neu, M.E., Hassan, Y., Verspohl, J., Beyerbach, M. and Kamphues, J. (2009) Effects of dietary additives (potassium diformate/organic acids) as well as influences of grinding intensity (coarse/fine) of diets for weaned piglets experimentally infected with *Salmonella* Derby or *Escherichia coli*. *J Anim Physiol Anim Nutr (Berl)* **93**, 350-358.

Ter Meulen, J. (2011) Monoclonal antibodies in infectious diseases: clinical pipeline in 2011. *Infect Dis Clin North Am* **25**, 789-802.

Thuenemann, E.C. and Lomonossoff, G.P. (2010) Transient Expression of Complex Heteromeric Bluetongue Virus-like Particles. In: *Molecular Farming:Plant as a Production Platform for High Value Proteins (WG2 and WG 3 Meeting)*. Hotel Wageningse Berg, Wageningen, The Netherland.

Tiller, T., Meffre, E., Yurasov, S., Tsuiji, M., Nussenzweig, M.C. and Wardemann, H. (2008) Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* **329**, 112-124.

Transue, T.R., De Genst, E., Ghahroudi, M.A., Wyns, L. and Muyldermans, S. (1998) Camel single-domain antibody inhibits enzyme by mimicking carbohydrate substrate. *Proteins-Structure Function and Genetics* **32**, 515-522.

Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J.P. and Katinger, H. (1996) Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J Virol* **70**, 1100-1108.

van Beers-Schreurs, H.M., Vellenga, L., Wensing, T. and Breukink, H.J. (1992) The pathogenesis of the post-weaning syndrome in weaned piglets: a review. *Vet Q* **14**, 29-34.

Van Daele, I., Gonzalez, N., Vercauteren, I., de Smet, L., Inzé, D., Roldán-Ruiz, I. and Vuylsteke, M. (2012) A comparative study of seed yield parameters in Arabidopsis thaliana mutants and transgenics. *Plant Biotechnol J* **10**, 488-500.

Van den Broeck, W., Cox, E. and Goddeeris, B.M. (1999a) Induction of immune responses in pigs following oral administration of purified F4 fimbriae. *Vaccine* **17**, 2020-2029.

Van den Broeck, W., Cox, E. and Goddeeris, B.M. (1999b) Receptor-dependent immune responses in pigs after oral immunization with F4 fimbriae. *Infect Immun* **67**, 520-526.

Van den Broeck, W., Cox, E., Oudega, B. and Goddeeris, B.M. (2000) The F4 fimbrial antigen of *Escherichia coli* and its receptors. *Vet Microbiol* **71**, 223-244.

Van der Stede, Y., Cox, E., Verdonck, F., Vancaeneghem, S. and Goddeeris, B.M. (2003) Reduced faecal excretion of F4(+)-E. coli by the intramuscular immunisation of suckling piglets by the addition of 1 alpha,25-dihydroxyvitamin D-3 or CpG-oligodeoxynucleotides. *Vaccine* **21**, 1023-1032.

Van Droogenbroeck, B., Cao, J., Stadlmann, J., Altmann, F., Colanesi, S., Hillmer, S., Robinson, D.G., Van Lerberge, E., Terryn, N., Van Montagu, M., Liang, M., Depicker, A. and De Jaeger, G. (2007) Aberrant localization and underglycosylation of highly accumulating single-chain Fv-Fc antibodies in transgenic Arabidopsis seeds. *Proc Natl Acad Sci U S A* **104**, 1430-1435.

Van Droogenbroeck, B., De Wilde, K. and Depicker, A. (2009) Production of antibody fragments in Arabidopsis seeds. *Methods Mol Biol* **483**, 89-101.

Van Molle, I., Moonens, K., Buts, L., Garcia-Pino, A., Panjikar, S., Wyns, L., De Greve, H. and Bouckaert, J. (2009) The F4 fimbrial chaperone FaeE is stable as a monomer that does not require self-capping of its pilin-interactive surfaces. *Acta Crystallogr D Biol Crystallogr* **65**, 411-420.

van Zijderveld, F.G., Anakotta, J., Brouwers, R.A., van Zijderveld, A.M., Bakker, D. and de Graaf, F.K. (1990) Epitope analysis of the F4 (K88) fimbrial antigen complex of enterotoxigenic *Escherichia coli* by using monoclonal antibodies. *Infect Immun* **58**, 1870-1878.

Vanbeersschreurs, H.M.G., Vellenga, L., Wensing, T. and Breukink, H.J. (1992) The pathogenesis of the post-weaning syndrome in weaned piglets - a review. *Vet Q* **14**, 29-34.

Verdonck, F., Cox, E. and Goddeeris, B.M. (2004a) F4 fimbriae expressed by porcine enterotoxigenic *Escherichia coli*, an example of an eccentric fimbrial system? *J Mol Microbiol Biotechnol* **7**, 155-169.

Verdonck, F., Cox, E., Van der Stede, Y. and Goddeeris, B.M. (2004b) Oral immunization of piglets with recombinant F4 fimbrial adhesin FaeG monomers induces a mucosal and systemic F4-specific immune response. *Vaccine* **22**, 4291-4299.

Vianna, G.R., Cunha, N.B., Murad, A.M. and Rech, E.L. (2011) Soybeans as bioreactors for biopharmaceuticals and industrial proteins. *Gen Mol Res* **10**, 1733-1752.

Vila, B., Peris, S., Calafat, F., Fontgibell, A., Esteve-Garcia, E. and Brufau, J. (2010) Strategies of use of a specific immunoglobulin-rich egg yolk powder in weaning piglets. *Livestock Science* **134**, 228-231.

Voinnet, O., Rivas, S., Mestre, P. and Baulcombe, D. (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* **33**, 949-956.

Vondruskova, H., Slamova, R., Trckova, M., Zraly, Z. and Pavlik, I. (2010) Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: a review. *Veterinarni Medicina* **55**, 199-224.

Waheed, M.T., Thoenes, N., Mueller, M., Hassan, S.W., Razavi, N.M., Loessl, E., Kaul, H.-P. and Loessl, A.G. (2011) Transplastomic expression of a modified human papillomavirus L1 protein leading to the assembly of capsomeres in tobacco: a step towards cost-effective second-generation vaccines. *Transgenic Res* **20**, 271-282.

Warnecke, T. and Gill, R.T. (2005) Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications. *Microb Cell Fact* **4**, 25.

Webster, D.E. and Thomas, M.C. (2012) Post-translational modification of plant-made foreign proteins; glycosylation and beyond. *Biotechnol Adv* **30**, 410-418.

Werner, S., Marillonnet, S., Hause, G., Klimyuk, V. and Gleba, Y. (2006) Immunoabsorbent nanoparticles based on a tobamovirus displaying protein A. *Proc Natl Acad Sci U S A* **103**, 17678-17683.

Wesolowski, J., Alzogaray, V., Reyelt, J., Unger, M., Juarez, K., Urrutia, M., Cauerhff, A., Danquah, W., Rissiek, B., Scheuplein, F., Schwarz, N., Adriouch, S., Boyer, O., Seman, M., Licea, A., Serreze, D.V., Goldbaum, F.A., Haag, F. and Koch-Nolte, F. (2009) Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Med Microbiol Immunol* **198**, 157-174.

Whaley, K.J., Hiatt, A. and Zeitlin, L. (2011) Emerging antibody products and Nicotiana manufacturing. *Hum Vaccin* **7**, 349-356.

Wieland, W.H., Orzaez, D., Lammers, A., Parmentier, H.K., Verstegen, M.W. and Schots, A. (2004) A functional polymeric immunoglobulin receptor in chicken (Gallus gallus) indicates ancient role of secretory IgA in mucosal immunity. *Biochem J* **380**, 669-676.

Willemsen, P.T. and de Graaf, F.K. (1992) Age and serotype dependent binding of K88 fimbriae to porcine intestinal receptors. *Microb Pathog* **12**, 367-375.

Wilson, J.A., Hevey, M., Bakken, R., Guest, S., Bray, M., Schmaljohn, A.L. and Hart, M.K. (2000) Epitopes involved in antibody-mediated protection from Ebola virus. *Science* **287**, 1664-1666.

Wilson, M.R. and Svendsen, J. (1971) Immunity to *Escherichia coli* in pigs - role of milk in protective immunity to *e. coli* enteritis. *Can J Comp Med* **35**, 239-243.

Winter, G., Griffiths, A.D., Hawkins, R.E. and Hoogenboom, H.R. (1994) Making antibodies by phage display technology. *Annu Rev Immunol* **12**, 433-455.

Wu, X.Y., Chapman, T., Trott, D.J., Bettelheim, K., Do, T.N., Driesen, S., Walker, M.J. and Chin, J. (2007) Comparative analysis of virulence genes, genetic diversity, and phylogeny of commensal and enterotoxigenic *Escherichia coli* isolates from weaned pigs. *Appl Environ Microbiol* **73**, 83-91.

Wurm, F.M. (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol* **22**, 1393-1398.

Wycoff, K.L. (2005) Secretory IgA antibodies from plants. *Curr Pharm Des* **11**, 2429-2437.

Xu, J., Dolan, M.C., Medrano, G., Cramer, C.L. and Weathers, P.J. (2011a) Green factory: Plants as bioproduction platforms for recombinant proteins. *Biotechnol Adv*, Epub ahead of print.

Xu, J., Ge, X. and Dolan, M.C. (2011b) Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. *Biotechnol Adv* **29**, 278-299.

Yokoyama, H., Peralta, R.C., Diaz, R., Sendo, S., Ikemori, Y. and Kodama, Y. (1992) Passive protective effect of chicken egg yolk immunoglobulins against experimental enterotoxigenic *Escherichia coli* infection in neonatal piglets. *Infect Immun* **60**, 998-1007.

Zeitlin, L., Cone, R.A., Moench, T.R. and Whaley, K.J. (2000) Preventing infectious disease with passive immunization. *Microb Infect* **2**, 701-708.

Zeitlin, L., Cone, R.A. and Whaley, K.J. (1999) Using monoclonal antibodies to prevent mucosal transmission of epidemic infectious diseases. *Emerging Infect Dis* **5**, 54-64.

Zhang, S., Wang, Z. and Wang, H. (2012) Maternal immunity in fish. *Dev Comp Immunol*, Epub ahead of print.

Zimmermann, J., Saalbach, I., Jahn, D., Giersberg, M., Haehnel, S., Wedel, J., Macek, J., Zoufal, K., Gluender, G., Falkenburg, D. and Kipriyanov, S.M. (2009) Antibody expressing pea seeds as fodder for prevention of gastrointestinal parasitic infections in chickens. *BMC Biotechnol* **9**, Epub.

Patent

Patent

Application no. EP 12182774.5

Protective Anti-ETEC Antibody

Abstract

The present invention relates to an antibody that can protect against ETEC infection in a passive immunization set up. More specifically it relates to a VHH grafted on an IgA scaffold, and produced in plant seeds. When the seeds are given in food or feed, the subject is protected against ETEC infection.

Claims

1. A fusion protein comprising an anti ETEC VHH fused to an IgA Fc domain.
2. The fusion protein according to claim 1 wherein said anti ETEC VHH is selected from the group consisting of an anti $F4^+$ ETEC VHH and an anti $F18^+$ ETEC VHH.
3. The fusion protein according to claim 2 wherein said anti ETEC VHH is specific for the FaeG domain of the *E. coli* F4 fimbriae.
4. The fusion protein according to claim 2 wherein said anti ETEC VHH is specific for the FedF domain of the *E. coli* F18 fimbriae.
5. The fusion protein according to any of the previous claims, wherein said IgA is a porcine IgA.
6. The fusion protein according to claim 5, wherein said porcine IgA is IgA^b.
7. A protein complex, comprising a fusion protein according to any of the claims 1-6, and a J chain and/or a secretory component chain.
8. A nucleic acid, encoding a fusion protein according to any of the previous claims.
9. The nucleic acid according to claim 7, wherein said nucleic acid comprises SEQ ID N° 1, SEQ ID N°2, SEQ ID N° 3 or SEQ ID N° 4.
10. A transgenic plant, expressing a fusion protein according to any of the

claims 1-6.

11. The transgenic plant according to claim 10, further expressing a J chain and/or a secretory component chain.

12. The transgenic plant according to claim 10 or 11, wherein said expression is seed specific.

13. The use of a fusion protein according to any of the claims 1-6 for the manufacture of a medicament for the treatment of ETEC.

14. A fusion protein according to any of the claim 1-6 for use in treatment of ETEC.

15. A transgenic plant, according to any of the claims 10-12, for use in treatment of ETEC.

16. The use of a transgenic plant according to claim 13, wherein the seeds are used in food or feed.

17. The use of transgenic plant seeds, comprising a fusion protein according to any of the claims 1-6, for the manufacture of a feed for the treatment of ETEC.

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Appendix

Appendix Table 1: Segregation analysis on the T2 seeds expressing VHH-IgG for determination of single locus insertions

	Km					For 3:1 ratio				
	Transformant	Resistant	Sensitive	Non-Germinated	Total germinated	expected resitant	expected sensitive	Chi square value	Locus	Ratio
	example 1 locus	48	16	0	64	48	16	0	1	3
	example 2 loci	60	4	0	64	48	16	12	x	15
1	V1A 13	11	16	37	27	20	6,8	17	x	0,7
2	V1A18	29	14	21	43	32	11	1,3	1	2,1
3	V1CA11	15	7	42	22	17	5,5	0,5	1	2,1
4	V1CA28	36	9	19	45	34	11	0,6	1	4
5	V2A17	32	9	23	41	31	10	0,2	1	3,6
6	V2A7	17	2	45	19	14	4,8	2,1	1	8,5
7	V3A 38	45	10	9	55	41	14	1,4	1	4,5
8	V3A 51	33	8	23	41	31	10	0,7	1	4,1
9	V3A13	25	6	33	31	23	7,8	0,5	1	4,2
10	V3A29	33	17	14	50	38	13	2,2	1	1,9
11	V4A 30	29	11	24	40	30	10	0,1	1	2,6
12	V4A 36	32	7	25	39	29	9,8	1	1	4,6
13	V4A 4	35	10	19	45	34	11	0,2	1	3,5
14	V4A8	42	2	20	44	33	11	9,8	x	21
15	V1cA 38	39	10	15	49	37	12	0,6	1	3,9

The T2 seeds of high expressing transformants were used for segregation analysis to determine plants with single locus insertion. Sixty-four seeds from each transformant plant were sown on kanamycin (km) containing medium, the number of resistant and sensitive plantlets were counted after 3 weeks of germination and also number of non-germinated seeds were counted. The ratio of resistant to sensitive was determined, and using Chi square test the transformants having 3:1 segregation ration of resistant to sensitive plants indicative of single locus insertion was statistically determined. In the above table plants having insertion at multiple loci are indicated by 'x'.

Appendix Table 2: Segregation analysis on the T2 seeds expressing sSIgA and dIgA (two selection markers) for determination of single locus insertions

Km					For 3:1 ratio					PPT				For 3:1 ratio					Km + PPT				For 3:1 ratio				
Transformant	Resistant	Sensitive	Non-germinated	Total germinated	Expected resitant	Expected sensitive	Chi square value	Locus	Ratio	Resistant	Sensitive	Non-Germinated	Total germinated	Expected resitant	Expected sensitive	Chi square value	Locus	Ratio	Resistant	Sensitive	Non-Germinated	Total germinated	Expected resitant	Expected sensitive	Chi square value	Locus	Ratio
example 1 locus	48	16	0	64	48	16	0	1	3	48	16	0	64	48	16	0	1	3	48	16	0	64	48	16	0	1	3
example 2 loci	60	4	0	64	48	16	12	x	15	60	4	0	64	48	16	12	x	15	60	4	0	64	48	16	12	x	15
V1A 2	36	3	25	39	29	9,8	6,2	x	12	36	3	25	39	29	9,8	6,2	x	12	37	5	22	42	32	11	3,8	x	7,4
V1A 8	30	13	21	43	32	11	0,6	1	2,3	30	13	21	43	32	11	0,6	1	2,3	30	21	13	51	38	13	7,1	x	1,4
V1cA 10	29	14	21	43	32	11	1,3	1	2,1	40	7	17	47	35	12	2,6	1	5,7	31	19	14	50	38	13	4,5	x	1,6
V1cA 23	40	2	22	42	32	11	9,2	x	20	37	0	27	37	28	9,3	12	x		37	1	26	38	29	9,5	10	x	37
V1cA 27	44	7	13	51	38	13	3,5	1	6,3	40	4	20	44	33	11	5,9	x	10	58	0	6	58	44	15	19	x	
V1cA 36	17	1	46	18	14	4,5	3,6	1	17	17	0	47	17	13	4,3	5,7	x		25	2	37	27	20	6,8	4,5	x	13
V2A 14	42	5	17	47	35	12	5,2	x	8,4	43	4	17	47	35	12	6,8	x	11	46	13	5	59	44	15	0,3	1	3,5
V2A 42	35	12	17	47	35	12	0	1	2,9	46	0	18	46	35	12	15	x		31	18	15	49	37	12	3,6	1	1,7
V2A 8	44	2	18	46	35	12	10	x	22	45	7	12	52	39	13	3,7	1	6,4	45	10	9	55	41	14	1,4	1	4,5
V3A 1	41	12	11	53	40	13	0,2	1	3,4	37	5	22	42	32	11	3,8	x	7,4	40	7	17	47	35	12	2,6	1	5,7
V3A 10	54	0	10	54	41	14	18	x		43	0	21	43	32	11	14	x		51	4	9	55	41	14	9,2	x	13
V3A 12	27	6	31	33	25	8,3	0,8	1	4,5	22	17	25	39	29	9,8	7,2	x	1,3	30	13	21	43	32	11	0,6	1	2,3
V3A 39	42	3	19	45	34	11	8,1	x	14	32	2	30	34	26	8,5	6,6	x	16	39	5	20	44	33	11	4,4	x	7,8
V3A 4	54	4	6	58	44	15	10	x	14	54	3	7	57	43	14	12	x	18	50	2	12	52	39	13	12	x	25
V3A 40	42	15	7	57	43	14	0,1	1	2,8	44	12	8	56	42	14	0,4	1	3,7	50	13	1	63	47	16	0,6	1	3,8
V3A 43	38	12	14	50	38	13	0	1	3,2	37	15	12	52	39	13	0,4	1	2,5	29	21	14	50	38	13	7,7	x	1,4
V3A 44	53	2	9	55	41	14	13	x	27	57	1	6	58	44	15	17	x	57	45	5	14	50	38	13	6	x	9
V3A 8	36	12	16	48	36	12	0	1	3	40	13	11	53	40	13	0	1	3,1	42	12	10	54	41	14	0,2	1	3,5
V4A 11	58	2	4	60	45	15	15	x	29	47	0	17	47	35	12	16	x		44	6	14	50	38	13	4,5	x	7,3
V4A 7	44	18	2	62	47	16	0,5	1	2,4	31	14	19	45	34	11	0,9	1	2,2	33	7	24	40	30	10	1,2	1	4,7

The segregation analysis was done for the listed set of transformants expressing high amounts of complexes either dIgA or sIgA. For each of the transformant 64 T2 seeds were sown on Kanamycin (Km) containing medium, 64 seeds were sown on phosphinothricin (PPT) containing medium, while another 64 seeds were sown on medium containing both kanamycin and phosphinothricin (Km+PPT), to determine the segregation pattern of plants with VHH-IgA or SC bearing T-DNA (both have nptII gene) (left panel), J chain (middle panel), and the ones having all the three genes (right panel). The number of resistant and sensitive plantlets was counted after 3 weeks of germination and also number of non-germinated seeds were counted from each plate. The ratio of resistant to sensitive was determined, and using Chi square test the transformants having 3:1 segregation ration of resistant to sensitive plants indicative of single locus insertion was statistically determined. In the above table plants having insertion at multiple loci are indicated by 'x'.

Acknowledgements

The PhD reel……

It is Thursday the 20th September, 8 days left for the public defence, nearly there…. Oddly, I have this strong inclination to look back……. I see days pass by in front of my eyes, like watching re-telecast of my favourite tele series. Its amazing how this journey of the last 4 years has been a pomegranate of precious moments, and I have deepest regards, respect and gratitude toward all who contributed to making this journey priceless.

It all began with..

'Episode 1- The opportunity'

I was adamantly seeking PhD position in the field of Molecular Farming, something I fancied since before my bachelors. My journey through classical botany and human virology got me to the **VIB's International PhD program**, which turned out to be the perfect matchmaker for me. I am really thankful to everyone at the VIB headquarters who sifted out my application and introduced me to my 'guru'- **Ann** (Ann Depicker). Ann accepted me as her PhD student and provided me with the opportunity to make this difference. Ann, as a teacher/ mentor/ guru/ promoter you have given me so much and for that I shall be always indebted. Thank you so much for being there and for supporting me and my ideas. I really appreciate your point of view, more so, because it is distinct and always shines light from a very unique facet. Your keen assessment has been a bundle of learning opportunity for me, helping me to be better— refine and refine myself. Thanks also for the encouragement that you provided, both in the lab when it came to trying out radical ideas, devising weird contraption or presenting at conferences and collaborating with peers. It's hard to pen down a précis of your contribution to my development as a scientist, but I can assure you that your mark will linger on. Forever, I shall be known as your once PhD student, and I take pride in that affiliation. Thank you.

'Episode 2- La_y-ma_n in the big town'

Before I could get my feet wet into the subject I found myself drenched in Brussels (literally). If Ann was the steering wheel of my PhD, then **Henri** (Henri De Greve) was the ignition. Henri, got me kick started into the topic with panning of nanobodies from lama immune library. He introduced me to my mentor **Els** (Els Pardon), thanks to the able and very structured guidance of whose; I had the ideal Nanobodies in no time. Thank you Els.

I remember one of those first days, **Henri** not just showed me around his lab, but also pleasantly surprised me when he actually took the pipet and showed me the agglutination protocol. Henri, you haven't ceased to pleasantly surprise me since that day on. You have always been a phone call away, you promptly replied to my mails even in the wee hours. You were always enthusiastic and as we say in India 'ready on one foot' to come down to Ghent for any meeting or guidance that I needed. Your constant support and backing was always motivating. I am particularly thankful for your coaxing during the final leg of my thesis writing, which helped in achieving what seemed like a herculean task of writing thesis in a month and a half.

'Episode 3- A greenhouse with 200 gardeners'

It was in a talk that I once heard, "One's postdoctoral career is his own responsibility but the credit of your PhD goes to your department". Those words though percolated, its true wisdom only dawned on me later. I am so thankful to the entire department of Plants Systems Biology for presenting this unique environment to excel. Thank you, **Dirk** (Dirk Inzé) for backing up our project, thanks also to the entire **management council of the PSB** (Dirk Inzé, Yves Van de Peer, Wout Boerjan, Lieven De Veylder, Moritz Nowack, Geert De Jaeger and Christine Tiré) for supporting our project

and helping us to bring it to the brim of the pipeline for valorisation. Your ideology and driving force for translational research, has pistoned our crankshaft to churn exciting ideas. I am grateful for the state-of-the-art facility that has been provided by the unique sisterhood of the **Ghent University, the VIB and the PSB.**
The department wouldn't have been this marvellous (and that is not just me bragging, the review board said so) without it's personnel who are dedicated, passionate and not to forget that special ingredient— slightly whimsical. Thank you all for making PSB such a vibrant and interesting environment to work in. One of the best things about the **PSB crew** is that I could just jump from my chair and scout out for any help; and the apt trick or advice is just minutes away. Thank you everyone, whose door I knocked, more often that you think for ridiculous sounding requests. Some of which sounded like "Where are the ferules, can you show me how to pack the column and connect to peristaltic pump (and then soon) can I dismantle the old peristaltic pump to give it an upgrade? **Geert** (Geert Persiau), thanks for sharing your tricks of protein purification, and for being ever so helpful. I bothered Bernard with questions like "Could you order one meter of nylon mesh, 2 coffee grinders, one Braun hand blender, one Kenwood cake batter mixer and can I get costume made Arasheets? Also could you get me an anti-static gun?" Thanks **Bernard** (Bernard Vanassche) for putting up with my order requests, I know I made you look into unconventional catalogues, but that never discouraged you. You went through lengths to help me. I am still curious, where did you find the anti-static gun? In an old vinyl record store, is it? Speaking of unconventional request reminds me of **Kristof** (Kristof Verleye) and his enthusiasm. Before I could wrap up my head about the idea of sawing one of the yellow bins, he had half the contraption in front of me. I shall always appreciate your enthusiasm and your skills. I dedicate the "veiled-bin" harvesting contraption to you (for the other PSBiets this contraption is that giant soup bowl that you often saw me carrying around). Thanks also to my anonymous seed donors, who spare some of their seeds for my experiment, it all added up to quiet a bit in the end (Chuckle! I know that last sentence sounds dicey).

Thank you **Mieke** (Mieke Van Lijsebettens) for your enthusiasm and your willingness to let me grow kilograms of Arabidopsis in the greenhouse. Thank you **Nico** (Nico Smet) **and Miguel** (Miguel Riobello) for taking such good care of my 'mini-farm'. I came into PSB with a black thumb; it's you guys and the nudging from my fellow lab mates that my thumb is sort of turning shades of green…keeping fingers crossed for the soybean now. Thanks to **Jackie**, for the autoclaving service, **Nancy** (Nancy Helderwert) for the media request. The **IT team** or should I say the *Council of Elrond*, thanks for your prompt service of the highest efficiency, your tricks are in no way les than wizardry, keep up the good magic. May the force of the server be with you. Thanks **Wilson** (Wilson Ardiles) for all sequencing requests. **Jenny** (Jenny Russinova), the one day you sent Niloufer to call me, I thought I am going to be hammered for being one of the pranksters to turn your office pink, but to my surprise you treated me with some lovely pastry. Thank you, thanks for being a sport, and thanks for the kind words and the support that you always share. **Camilla** (Camilla Betti) I often picked your brain along with Niloufer's, thanks for sharing all your protein advice, and the interesting fashion updates (nice to catch up trends). Thanks **Hilde** (Hilde Nelissen) for all the help with the doctoral school administration, for your much-needed reminders and for making sure all went well. Thanks also to our front office, **Christine** (Christine Tire) **Diane** (Diane Hermie), **Sophie** (Sophie Maebe), **Nathalie** (Nathalie Vanden Hautte) and **Delphine** (Delphine Verspell), for all the admin help and the various favours.

'Episode 4- Cup of T.T.T'
My special thanks goes to my group (Sylvie, Annelies, Kirsten, Thomas, Robin, Jonah, Els, Nancy, Lien, Rim and Ann) you have enriched my life both in the lab and at occasions when we strayed outside our scientific perimeters. O! I have a whole bunch of excellent

memories just pouring out….. the jokes, fun times, the parties, the lunch, the fries, the lab meetings (and the very longgggg meetings), the funny pranks in the lab…uff! I have bucket loads of moments to thank you all for.

Sylvie (Sylvie De Buck), thanks for the numerous things you have done for me— harvesting, driving, reading my manuscripts, etc. etc. but above all, thanks for being my soundboard. I have always valued your opinion and I am grateful that I could learn from your expertise. Whenever I was not sure of the chirpy waters you helped me sail perfectly, like a vigilant and caring captain. Dank u! **Kirsten** (Kirsten De Wilde), I am always been your 'not so silent' admirer (wink!!). I have been impressed, with your organisational skills, your zeal for perfection, your warmth as a person and your people skills. It was joyous and one of the most special memories sharing the lab booth with you— working with you, discussing lab and beyond. You were my agony aunt, buddy, fashionista, and one of the most fun people I will ever know. Thanks for being an inspirational colleague, neighbour and friend. **Annelies,** (Annelies De Paepe) half the time I came to your desk was to ask a question and the other time it was to share something exciting. I am glad that you always reciprocated with the equal excitement, always helpful and so cool. Yup! Cool in a 'Zen' sort of way. I learn quiet somethings from your lab results and recently I also learnt some cooking from you… the lasagne was a success more than once. **Els** (Els Van Lerberge) **and Jonah** (Jonah Nolf) the two managers of our lab, you guys take care of a lot of things so that we do not have to bother. Things like maintaining the cryo stocks, ordering supplies etc that we take for granted, thanks for running our system processes so that we can concentrate on our experiments. Especial thanks to both of you for helping me out with the harvesting of the 'fields' not just in our greenhouse, but in Brussels and at Melle. Thanks for being so kind; you guys have literally shed the sweat for my project. Jonah, I know you tried very hard to get me pink supplies, but alas! the manufacturers of lab supplies are not as thoughtful as you. **Thomas** (Thomas De Meyer) **Miguel** (Miguel Cardoso) **and Robin** (Robin Piron), it's been fun working with you guys. Thanks for the discussions, the refreshing chats and the jovial atmosphere. I wish you all the very best for your PhD. Thanks also goes to **Nicholas** (Nicholas Keirse), for the numerous western blots that you developed and quickly picked up the knack of quantifying the VHH-IgA bands. I must say it was not one of the easiest things to do as a student, but you faired well. **Hana** (Hana Hoffmeisterova), hats off to your hard working attitude, your support was like adding a booster battery to the high-throughput platform. In no time I had all the results I wanted. Thank you, for the lovely exchange experience and for tell me about Prague. You introduced me to plants and spices, the whereabouts of which I knew only to exist in supermarket, thanks. **Joline** (Jolien De Block) **and Joke** (Joke Boute), the honorary members of the TTT, thanks for lightning up the lunch breaks, social evenings and all the fun times.

'Episode 5- Save thy piglet!'

The idea of *in vivo* challenge experiment was extremely exciting, but little did I know what I was in for. Venturing into these exciting unforeseen alleys, **Prof. Cox** (Eric Cox)**, Prof. Goddeeris** (Bruno Goddeeris) and **Annelies** (Annelies Coddens) had become my shining beacon to guide me through. I would have been very lost with out your guidance. Thank you Prof. Cox for your enthusiasm and willingness to accept me in your lab, for being my promoter and for engaging in this rather grand experiment. Annelies, I had the opportunity to learn from your expertise, you trained me to work with a model organism, which was utterly new to me, thanks. I also appreciate that in spite of my naivety you were willing to take chances and incorporate my idea into the working of the experiment. Thank you so much for being such an excellent teacher, for your guidance, organisation and your meticulous analysis of the results and my manuscript.

For the ones who have worked with piglets before— will know; and the ones who haven't— well, just take my word for the enormity of the experiment (my muscles will

vouch). An experiment of this size is unthinkable without the experts, thanks to **Simone** (Simone Brabant), **Griet** (Griet De Smet), **Pieter** (Pieter Almgren) and **Rudy** (Rudy Cooman). All of you, never shied off from helping me, always cheered me on with a bright smile and taught me your tricks. My sincere salute to your hard work, I don't know how to stress that enough, thanks in leaps and bounds.

'Episode 6- Lets play farmer'

What does it need to grow kilograms of Arabidopsis? Space! Thanks to our friends and collaborators who could generously provide us with their greenhouse space. Thanks **Marc** (Marc Deloose) and **Bart** (Bart Von Droogenbroeck) for making this possible for us within the ILVO greenhouses. Thanks **Geert** (Geert Angenon) and **Martine** (Martine Claeys) for growing our plants in your greenhouse in Brussels and taking such good care of them. Cheers, and thank you so much for sharing the same passion as we do and for believing in Arabidopsis platform. It's because of your contribution that I could summarise the results in this manuscript.

Sam (Sam Millet), I sincerely appreciate you help, especially during the last few months with the formulation of nearly 100 kg of feed and the statistical analysis of the piglet experiment. Prior to meeting you I had no idea about the feed formulation, thanks for introducing me to this whole field of research. It was enlightening. Like some say, "What we eat, is what we are" the same holds true for the tiny piglets. **Sven** (Sven Arnouts) thanks for all the help and market data that you provided, thanks for the arrangements with the companies and for the flax seed. Also I would like to thank **Dirk** (Dirk Iserentant) for the help with patent filing, and special thanks for putting up with my verbose style of writing.

"Episode 7-Peas please"

Our collaborating partners from University of Rostock, **Inge** (Inge Broer) and **Heike** (Heike Mikschofsky) helped us in taking one step closer to valorisation. Thank you Inge for having me in your lab, it was very rewarding experience to participate in your lab meetings, thanks also for the amazing hospitality, I am always trying to replicate that chicken dish you cooked. Heike, Thank you, thank you so much. I am so glad to know you, to make friends with you. Thanks for teaching me about pea transformation and for transforming my constructs in pea. I have recently received the first seeds 'the little babies' hope they bear some great fruits. Thanks also for taking such good care of me whiles I was in Rostock, I shall always remember the experience. Hope to see both of you soon.

"Episode 8- Indian tea party "

My Indian gang- **Jacob, Tessa, Anju, Anagha, Niloufer, Dip-tea, Rahul, Sibu, Elisabeth and Clair** (yes, Clair- if you get letters from Indian Embassy then you are Indian)- Love you guys, thank you for being there with me through thick and thin. Thanks for being my Ghent family. I know I gave the expression 'lend me your ears' a whole new meaning but….. (let me not continue with that, I have picked your brains already too much). I just love our tea parties, where tea is optional and once our evening 'tea' kindles scientific discussion, well that's when even the physicist should watch their angular velocity and plummeting speed, because some people are like "I am going to fall…. but its ok". Dear readers, does this episode make any sense? No?, well that is the point, and trust me its very satisfying. Such Fun!! Such Fun!!

Niloufer, of all the "Bheja Fry " I ever had, yours is my favourite, thanks a million. I have learnt so, so much from you, yours has been a special contribution to my PhD, you have been my friend and my silent mentor, thanks also for the occasional 'kick on the butt…..' (might I say much needed). I could always count on you when I needed a push, or some food for that matter. Miss you! **Tessa**, many a times during my course in Ghent, I needed a punctuation, to reanalyse and kick back with full enthusiasm and spirits, thanks for

being that quintessential check post in my life. You and Niloufer inspire me, Thanks. **Anju and Anagha**, I so much crave for the Friday dinner-laundry nights, minus the laundry of course….

"Episode 9: Where is the sugar?"
Thanks **Nico** (Nico Callewaert), **Bieke** (Bieke Nagels) and **Francis** (Francis Santens), for your help with the glycan analysis and all the tips. I hope we can unravel the sugar mystery soon

"Episode 10- Special finale- writer's take"
I wouldn't have been at this stage of writing this acknowledgement, if it weren't for **Jurgen**, seriously, I would have still been making my figures. Thank you Jurgen for filling my book and my life with the vibrant happy colours, one to rival Desigual's summer collection. Thanks for making me feel like Patek Philippes's 22 carat, precious and always on your arm. **Ashuwini**-my best friend, no matter where I go in life, I know you shall be there as a strong mystical pillar who can make anything happen. Thanks for being my BFF, so proud to have you in my life. My dearest **Nanus and Nanijis**, thank you for always supporting the idea of my higher education. I am really glad that you will be there to toast my achievement. **Mumma**, the coolest person I know, you brought me up as your best friend, in my dreams you saw yours, and for my happiness you defined everything. Thank you so much mummy for letting me make all my decisions and my mistakes, but you were always there as a safety net. I dedicate this book and the accomplishment to you. **Sahib**, thanks for your support throughout these 4 years, for believing in me and for providing me with the much-needed timely revision of mumma's philosophical wisdom. And most importantly, for approval of my shoes and my outfit ;-).

Lastly, I would like to thank all members of my examination commission for their time and patience to go through my thesis. Thank you everyone for the critical evaluation of my work and for the suggestion during the internal defence, much appreciate it. It was truly gratifying to receive your interest and enthusiasm about my thesis,

Thank you so much,

Cheers, to you all,

Vikram

Curriculum Vitae

VIRDI VIKRAM

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Gent, Belgium

Personal Information

Gender:	Male
Date of birth:	15th December 1984
Place of birth:	Pune, India
Nationality:	Indian
Residential Address:	Cotton Island, 97 Edward Pynaertkaai, apartment 0401, 9000 Gent, Belgium.
Cellular Phone No.:	0032 471743749
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Educational Qualification

PhD (2008-2012)

Title of the thesis: Oral passive immunization of weaned piglets against F4-positive enterotoxigenic *Escherichia coli* by seed produced antibodies
At the Department of Plant Systems Biology (VIB) Ghent University
Supervisors: Prof. Ann Depicker, Prof. Eric Cox and Prof. Henri De Greve

M.Sc. (Virology) (2005-2007)

Title of the thesis: Detection and Genotyping of Human Respiratory Syncytial Virus from Clinical Specimen
GPA: 7.79/10, Grade: 'O'-Outstanding
At the National Institute of Virology, University of Pune, India
Supervisors: Dr. M.S. Chaddha and Dr. R.G. Damle

B.sc. (Botany) (2002-2005)

Grade: First Class with Distinction (82.25%)
At the Fergusson College, University of Pune, India

Experience

- Part of the organization committee for the COST meeting on Molecular Farming, Gent, Belgium (Sep 2011)

- Supervised thesis: Molecular Characterization and Pre-trial Evaluation of Plant made Anti-ETEC Antibodies, Nicholas Keirse, Vikram Virdi, Sylvie De Buck, and Ann Depicker. Technical Bachelors thesis submitted at the Hogeschool Gent. (Feb to June 2011)

- Assisted as coach for Dr. Jean-Luc Doumount's course- 'Effective scientific communication' at the Gent University. (May-June 2011)

- Member of the organization committee for the International PhD symposium, Leuven, Belgium (Oct 2010)

- Conducted course 'Introduction to Bioinformatics' at the MITCON e-School. (Oct –Dec' 2007; June 2008)

- Conducted lectures twice in 'Fundamentals of PCR technology' as guest lecturer at MITCON, Biotechnology Lab, Pune, India and at undergraduate college affiliated to university of Pune. (June 2008)

Additional research experience

- Evaluation of circulating genotypes of Human Respiratory Syncytial Virus in tropical India during summer epidemic of 2006 (May 2007 to Feb 2008)

Clinical experience

- Clinical Clerkship at the King Edward Memorial Hospital, Pune. India (May 2006 to June 2006-)

Training sessions attended

- Laboratory animal Course, Ghent University (certified for working with Lab animals in Belgium) (Oct 2011)

- Effective scientific communication course by Dr. Jean-luc Doumont, Ghent University (Nov 2010)

- Protein structure analysis training, coach Dr. Joachim Jacob and Dr. Stephane Plaisance, VIB Research Training Course (June 2010)

- Nanobody and protein production training, organized by Prof. Serge Muyldermans and Dr. Gholamreza Hassanzadeh Ghassabeh, VIB Research Training Course (May 2010)

- Microscopy and imaging techniques, organized by Dr. Chris Guerin VIB Research Training Course (Nov 2009)

Presentations, Publication and Posters

- Immediate passive protection of farm animals against enteric diseases by in feed production of antibodies. (March 2012)
  At the UGent Ph.D students symposium, Gent, Belgium

- Passive immunization of piglets against post weaning diarrhoea via anti-ETEC antibodies produced in seeds. (September 2011)
  At the Molecular farming COST action meeting, Gent, Belgium

- Recombinant plant made antibodies against fimbriae of enterotoxigenic *Escherichia coli* inhibit in vitro binding to villous enterocytes. (July 2011)

- At the E coli and Mucosal Immune System conference, Gent, Belgium
  Seed produced anti ETEC antibodies for oral passive immunisation of piglets against post weaning diarrhoea, (June 2011)
  At the Plant-Based Vaccine and Antibodies Conference, Porto, Portugal

- Seed produced anti ETEC antibodies for oral passive immunisation of piglets against post weaning diarrhoea, Vikram Virdi, Sylvie De Buck, Hana Hoffmeisterova, Annelies Coddens, Eric Cox, Henri De Greve and Ann Depicker. PBVA conference, Porto, Portugal (June 2011) (poster)

- Vikram Virdi, Kavita Bose, Bhavishya Ravindran, Minakshi Mahajan. 2005. 'Petrocrops' Eco. Env. & Cons. 11(2): 2005; pp. (311-314)

- Petro-crops, crops that yield biofuel, awarded gold medal for this presentation (December 2003)
  At the Botany Fest, Nowrosjee Wadia College of Science, University of Pune, India

Language skills

English:	Fluent	(Reading, writing, and speaking)
Hindi:	Good	(Reading, writing and speaking)
Punjabi:	Fluent	(Speaking)
Marathi:	Average	(Reading, writing and speaking)
German:	Basic	

Hobbies and intrests

Theatre plays, craft, traveling and cultural studies.

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